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THE SIGNIFICANCE OF SURFACE AREA DETERMINATIONS

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THE selection of methods of measuring the surface area of animals is ordinarily made upon the basis of accuracy and of convenience. An accurate method is considered to be one that will give reproducible results, while it is, of course, obvious that methods adaptable to small animals may be entirely impracticable for large animals. However, whether the surface is determined by measuring the area of the removed hide, or of a mold fitted closely to the body, or by the mechanical integration of the area of the hide *in situ*, or by other means, the prevailing impression seems to be that the surface area of an animal is a definite measurement to which all good methods should approximate, and hence that all good methods are equivalent in the significance of the results obtained.

The area of a removed hide, if it can be spread out on a flat surface, either as removed or divided into a small number of pieces, and if it is not greatly extensible, may be readily measured, and on remeasuring, a satisfactory duplicate result may be obtained. This is true of cattle hides and of sheep hides. If the skin is greatly elastic it is not susceptible to this treatment. It has been the practice in this laboratory, in measuring the area of chicken skins, which are of this description, to stretch them before outlining them on paper. It is felt that this method was the only one capable of giving results even approximately reproducible, while at the same time it was realized that the results thus obtained were not comparable with results obtained on the skins of other animals, on which it is unnecessary to apply tension.

The extensibility of the hide of an animal when removed from the body, as well as the difficulty of making it lie flat upon a smooth surface, has induced many investigators to turn to other methods of determining surface area. Among these may be mentioned especially the making of molds

of one kind or another of the entire body or of a symmetrical part of the body, and the ingenious method of Brody of determining by a mechanical integrator, the area of the hide *in situ*. Such methods have given highly reproducible results and have contributed to the common belief that the surface area of an animal is a constant.

However, it seems obvious, when this proposition is critically examined, that it cannot be strictly true and may in fact be considerably in error. An animal may change its shape considerably by changing the position of its limbs, its head, and its trunk and each change in shape will occasion a change in surface area. The success of methods of determining directly the surface area of animals, and the close reproducibility of the results obtained, must depend upon the fact that a standard position of the body and its appendages is maintained throughout a series of measurements.

In order to ascertain the extent to which change in position will affect surface area, the surface area of a number of chickens and rats was determined by a mold method, described in the following paper, the position of the bodies being varied between two extremes. With the chickens, the accuracy of the method was first tested by cutting the gauze mold from the body in the median sagittal plane, the limbs being in the same position on the right and left side. In another series of trials, the limbs on one side of the body were placed in a position as nearly contracted as the method of putting on the mold permitted, while the limbs on the other side were fully extended. The areas of the two sides were then compared as before.

The results in the first series, in which both legs were contracted and both wings extended, are summarized in Table I.

TABLE I

THE SURFACE AREA OF THE RIGHT AND LEFT HALVES OF CHICKENS* WHEN BOTH LEGS ARE CONTRACTED AND BOTH WINGS ARE EXTENDED.

Bird No.	Sex	Body weight grams	Surface area of		Per cent difference
			Left side sq. cms.	Right side sq. cms.	
22	pullet	1074	483	491	1.64
24	cockerel	1799	646	659	1.99
25	cockerel	1978	693	707	2.00
26	cockerel	1458	580	580	0.00
27	cockerel	1653	628	625	0.48
28	cockerel	1841	651	641	1.55
29	cockerel	2142	728	729	0.14

* Exclusive of the shanks and feet and of the combs and wattles.

Evidently the method is capable of close duplication, within 2 per cent. However, if the position of the legs and wings is not symmetrical, the surface areas of the right and left halves may become appreciably different, as the results in Table II indicate.

TABLE II

THE SURFACE AREA OF THE RIGHT AND LEFT HALVES OF CHICKENS[1] WHEN THE LEG AND WING ON ONE SIDE ARE CONTRACTED AND ON THE OTHER EXTENDED

Bird No.	Sex	Body weight grams	Surface of		Per cent difference
			Left side sq. cms.	Right side sq. cms.	
23	pullet	1059	429[2]	478[3]	10.81
—[4]	—	—	396[3]	475[2]	18.16
24	cockerel	1799	613[2]	690[3]	11.82
25	cockerel	1978	698[3]	686[2]	1.73
26	cockerel	1458	546[3]	585[2]	6.90

[1] Exclusive of the shanks and feet and the comb and wattles.
[2] Leg and wing extended.
[3] Leg and wing contracted.
[4] Results from a second mold of Bird No. 23.

An explanation of the negative result for Bird No. 25 has not been revealed, but with the other birds, particularly the first two, there can be no doubt that the extension of leg and wing has increased the surface area of these appendages over that in the contracted position. However, variable results may be expected unless the extent to which leg or wing is contracted is standardized.

In this experiment with White Leghorn chickens no attempt was made to change the position of head, neck, or trunk. In the work on rats, the position of these members also was varied between two extremes. In the one position, the rat carcass was laid flat on its abdomen and the head and legs were extended to the fullest extent while the mold was being formed. This position is not unlike that sometimes assumed by a rat across the floor of its cage on a very warm day. In the other position, the carcass was placed upon its haunches and made to assume a partially crouched attitude by bending it over a glass feeding cup, tipped on its side. The limbs were partially contracted. Before applying the mold in these positions the hair was removed from the carcasses by immersing them for a few minutes in 10 per cent solution of barium sulfide.

Since the rats used in this study were of different sizes and ages, and since it has not been found satisfactory to take more than one mold of a carcass, because of an apparent shrinkage in the carcass after the first covering is removed, and of a change in the pliability of the skin due to the collodion used as a cement for the gauze covering, the areas obtained in each case have been compared with areas computed from Lee's formula (1) $S = 10.76W^{0.61} X\ 0.310/N_{obs}$, N_{obs} being Cowgill's "nutritive correction factor" obtained by dividing the cube root of the weight, in grams, by the length of body from nose to anus, expressed in centimeters. This formula was used in preference to the other formula suggested by Lee, since our rats were evidently in better nutritive condition, with values of N_{obs} ranging from 0.288 to 0.304.

In order to discover whether the mold method used in this work was essentially equivalent to Lee's method, by which the depilated skin is fixed, while on the carcass, by dipping into a nitro-cellulose lacquer, two rat carcasses were molded in a position similar to that used by Lee. The first rat, a male, weighed 126 grams, with a body length of 16.5 cms. and an N_{obs} of 0.304. The area of the mold was 213 sq. cms. and that predicted from Lee's formula was 210 sq. cms. The second rat, a female, weighed 214 grams and possessed a body length of 19.9 cms. In this case $N_{obs} =$ 0.301. The area of the mold was 304 sq. cms., as compared with a predicted area of 293 sq. cms. In the fomer case, the deviation between observed and calculated areas was 1.46 per cent and in the latter 3.62 per cent. It may be concluded that the two methods are essentially equivalent.

A number of rats were then molded in the two positions described above. The results obtained, and the estimated areas by Lee's formula are summarized and compared in Table III.

It seems clear that the surface area of the rats was markedly affected by the position in which the body was placed. With one exception, the 5 rats molded in the extended prone position gave areas considerably greater than those predicted by the formula of Lee. The average excess for the group was 5.77 per cent. All of the 4 rats molded in the crouched position possessed mold areas considerably less than the predicted areas, averaging −6.20 per cent. The average difference between the areas obtained in the two positions is thus practically 12 per cent. Furthermore, this difference is not due to wrinkling of the skin in the crouched position; it is a result of the change in shape of the carcass and the elasticity of the skin covering it.

1. Lee, M. O., Determination of the surface area of the white rat with its application to the expression of metabolic results. *Amer. Jour. Physiol.*, 1929, LXXXIX, 24.

The true carcass of the rat is very loosely enclosed in a skin which possesses a high degree of elasticity. This fact is well illustrated by such observations as the following. A male rat weighing 413 grams was skinned, and the area of the skinned carcass was found by the mold method to be 430 sq. cms. The unstretched skin possessed an area of 536 sq. cms. Only

TABLE III
THE SURFACE AREA OF RATS PLACED IN DIFFERENT POSITIONS

Rat No.	Sex	Body weight grams	Body length cms.	N_{obs}[1]	Surface area		Per cent difference
					Observed sq. cms.	Calculated sq. cms.	
Rats in the extended position							
3	f	185	19.8	.288	302	280	+ 7.28
9	m	149	18.1	.293	235	241	− 2.55
11	m	154	18.3	.293	266	246	+ 7.52
6	f	126	17.3	.290	247	220	+10.93
13	f	209	20.3	.293	318	300	+ 5.66
Rats in the crouched position							
7	f	138	17.8	.291	220	232	− 5.45
12	m	202	19.9	.295	271	288	− 6.27
10	m	198	19.6	.297	270	282	− 4.44
8	m	189	19.5	.294	255	277	− 8.63

[1] $N_{obs} = W^{\frac{1}{3}}/L$

a moderate degree of stretching would increase this area to 630 sq. cms. Similar results were obtained with other rats, so that it appears that the rat is provided with the means of changing its surface area within wide limits by changing the relative position and the shape of its trunk and appendages. That such changes in surface area actually result from change in the shape of the body, the experimental results cited above clearly prove. Even greater changes than those found would be expected if it had been possible to mold the rat carcass in a more contracted position than that chosen.

It may be concluded, therefore, that with chickens and rats, and presumably with other animals also, the surface area is not a definite measurement, but depends to a considerable extent upon the shape of the body, as determined by the position of the body trunk and its appendages. Hence, direct measurements of the surface area of animals placed in some definite position, although they may be closely reproducible and readily conformable to mathematical description, do not possess the definite interpretation ordinarily given them. For example, there is neither a

rational nor an empirical basis for assuming that the basal metabolism of an animal is more closely related to the surface area determined from a certain convenient position of the animal than to the surface area relating to any other position that the animal may naturally assume. And the possible differences between the surface areas of the same animal in different natural positions are not inconsiderable.

These considerations mean that the heat produced by an animal per square meter of body surface possesses no absolute meaning, but that it may still be a highly useful measurement for comparative purposes if the surface measurement for different animals is made by exactly the same method. Within any species it would appear that the basal heat produced per square meter of hide area is just as valuable and significant a measurement as the basal heat produced per square meter of surface area relative to some definite body position, provided the hide area is as accurately determinable as is the surface area. Both measurements can be determinable with accuracy only by the imposition of arbitrary conditions, relating in the one case to the tension, if any, that must be applied to the hide to obtain reproducible results, and in the other case to the position in which the animal must be placed for the most convenient measurement. The hide area of an animal, provided no considerable tension has been applied, may be considered to be a maximum surface area to which the body may attain by change of position. Evidently for animals of different species, the comparability of surface area determinations may be less close, due to the different character of the hide, or to other anatomical differences.

Needless to say, the method of eliminating the effect of differences in size of animal upon basal heat production by expressing the latter per unit of body area is just as valid as ever. Certainly the main value of such expressions is their comparability within the species. In formulas of the Meeh-Rubner type the particular value of the constant becomes thus a matter of indifference, while that of the exponent of the body weight is of first importance.

THE SURFACE AREA OF SINGLE COMB WHITE LEGHORN CHICKENS

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IN UNDERTAKING the determination of the surface area of a considerable number of animals by an exacting and time-consuming method, and in burdening the literature with a description of the results secured, one should have a definite conviction of the value and significance of surface area determinations. If the method of expressing basal metabolic rate with reference to a unit of surface area has only its popularity to commend it, as Benedict believes, the time devoted to surface area determinations is hardly time well spent.

The empirical basis of the so-called surface area law seems a sound one, since among a number of animals of the same species differing only in size, the basal heat output, when computed per square meter of body surface, presents a more uniform set of values than when computed per unit of weight. Thus, this method of reference is commonly considered to be the most satisfactory method of eliminating the effect of differences in size of body upon basal heat output. However, this practice carries no implication that no other factor than surface area affects basal heat output. It is a specious argument to urge against it the well known facts that age, sex, muscular development, nutritive condition, and various types of glandular malfunction may also affect basal metabolism; they would disturb any other method of relating basal metabolism to body size.

If a method is empirically sound, it should have some sort of a rational explanation, and not until such an explanation is at hand can the true significance of the method be appreciated and the utmost faith in its validity be felt. Obviously, surface area can be a factor in the determination of basal heat production only in so far as it determines the rate of heat loss from the body, and the necessity of any constant relation between surface area and basal heat production, if such there is, must relate to the necessity of maintaining within narrow limits a constant body temperature. But for warm-blooded animals there is a considerable range of environmental conditions within which the basal heat produced is approximately constant and greater than that needed to maintain body temperature. Hence, within this range heat loss and surface area are not determinants in basal heat output. But within this range, the requisite constancy of pro-

toplasmic temperature is not seriously threatened by environmental conditions. Outside this range, the mechanism for the preservation of a constant cellular temperature is subjected to greater and greater strain. Thus, for environmental temperatures lower than a certain critical value, the rate of heat production of the animal must be increased to keep pace with the increasing heat loss through the surface of the body. Also for environmental temperatures above body temperature, the evaporation of water, mainly from the skin, must be greatly increased, since it alone must bear the burden of heat excretion. Thus, under those environmental conditions threatening the continuation of animal life, the area of the body surface becomes an important factor in the regulation of body temperature.

For warm-blooded animals it may be considered that the basal heat production, in so far as it is related to size of body, is to a very large extent determined by the critical environment. At the critical temperature, the basal heat production must be equal to the minimum heat loss consistent with the maintenance of body temperature. This heat loss will depend upon the surface area of the body, the radiating capacity of the skin and its covering, and the normal body temperature. If all animals possessed the same critical temperature, they must possess the same basal heat production per unit of surface area, except for differences in normal body temperature and in the insulating properties of the skin and its covering. Differences in normal body temperature within the same species of warm-blooded animal are insignificant in this connection, and even among different species they amount to only a few degrees centigrade. Differences in the insulating properties of the skin and its covering may be great, but the more effective the skin is as an insulator of heat, the lower is the critical temperature of the animal, and hence the lower is the environmental temperature to which the basal heat production is adjusted. The net result will be a marked tendency, such as has been observed experimentally, for all animals, regardless of differences with respect to skin covering, body temperature, and critical temperature, to possess approximately the same adult basal heat production per unit of surface area. The effects of age, sex, muscular development, nutritive condition, and glandular functioning on basal metabolism are, of course, unrelated to size and surface area, and their existence does not vitiate, though it may obscure, the relation of surface area to the basal metabolic rate.

Thus, the characteristic features of warm-bloodedness in animals as they have been observed and reported, render inevitable a close relation between surface area and basal heat output. The relation possesses a

physical significance which is entirely ignored in Brody's recommendation (1) that basal metabolism be considered merely as a power function of body weight rather than as a function of body surface.

With the conviction that surface area is a real determinant in basal heat output, the surface areas of 25 Single Comb White Leghorn chickens, varying in body weight from 109 to 2142 grams, were measured in order to derive a formula by which surface area can be readily computed.

The birds were killed by bleeding and debraining and were then dry picked. They were then measured and laid out in a standard supine position with neck and wings extended and legs as nearly contracted as the method of molding permitted. The wings were pinned down in the desired position and the legs were supported on strings suspended from a laboratory ring stand. The comb and wattles were then cut off as were the ear lobes in the larger birds. The surface of the bird was then covered closely with strips of ordinary medical sterilized gauze, either 2 inches or 1 inch in width, which were made to adhere to the body and to each other as they were put in place by collodion applied with a brush. By varying the size of gauze and the length of the strip it was possible to cover all parts of the body regardless of their curvature. However, the shanks and feet were not covered. After the ventral part of the body was covered, the bird was turned over and covered on the dorsal side without changing the position of legs and wings. The completed mold was dry in one hour or less of standing, during which time a slight contraction of the gauze occurred, insuring a tight fit. In removing the mold from the body, it was first cut in two parts along the median sagittal line, and then was cut along the neck, wings, and legs as was found necessary for convenient removal. After removal from the body, the mold was cut up into pieces of such size and shape that they would lie flat, outlined with a pencil on a large sheet of paper, and their combined area determined with the planimeter. Depending upon the size of bird, it was found necessary to cut the mold into 17 to 50 pieces. The comb and wattles were also outlined and the area doubled, allowance being made in the case of the comb for the area of the surface of attachment to the head. The ear lobes, when large enough to require separate treatment, were outlined and measured, and allowance also made for the area of attachment. The area of the shanks and feet was determined by skinning one shank and foot, determining the area by cutting up, outlining, and applying the planimeter, and doubling this area.

Besides the live weight and surface area, three linear measurements were taken, *i.e.*, (a) the over-all length, from tail to tip of beak, (b) the rump-to-shoulder length, and (c) the circumference of thorax taken over the keel

and just behind the wings. On many of the birds, the picked, bled weight was also recorded. These measurements and weights are all contained in Table I.

TABLE I

THE BODY WEIGHTS, BODY MEASUREMENTS, AND SURFACE AREAS OF WHITE LEGHORN CHICKENS

Bird No.	Body weight grams	Surface area sq. cms.	Length over all cms.	Rump to shoulder cms.	Circumference cms.	Picked weight grams
8	110	227	18.0	7.4	11.0	—
9	109	220	18.0	7.4	10.5	—
11♂	235	376	25.0	9.1	14.0	—
12♂	341	526	27.5	11.1	15.0	—
13♂	449	618	29.0	11.8	17.0	—
14♂	555	731	33.0	13.2	18.0	490
15♂	578	781	33.5	13.2	19.5	504
16♀	668	795	35.5	13.7	19.5	570
21♀	840	908	39.5	16.1	22.5	712
18♀	984	1014	39.5	17.0	23.5	861
23♀	1059	1038	42.0	16.5	24.0	920
17♂	1072	1155	40.5	16.7	23.5	948
22♀	1074	1127	44.5	16.8	24.5	937
20♀	1109	1174	41.0	16.9	23.5	947
19♀	1213	1152	41.5	17.6	25.0	1095
5♀	1273	1172	40.5	16.0	24.5	1121
7♀	1329	1247	45.0	17.2	25.0	—
26♂	1458	1470	45.5	18.5	26.5	1270
6♀	1495	1469	46.0	—	29.5	—
10♀	1513	1435	47.0	18.2	27.0	—
27♂	1653	1602	48.0	18.9	28.5	1423
24♂	1799	1684	48.0	19.4	27.5	1612
28♂	1841	1612	48.0	18.0	28.0	1600
25♂	1978	1720	50.5	19.9	29.0	1725
29♂	2142	1894	49.5	21.3	30.0	1918

In attempting to fit a prediction formula to these measurements of surface area, it was realized that a close fit was hardly to be expected, because of a variable feather coat, which would affect body weight, but not body surface as measured from the picked carcass, and because of a variable growth of comb and wattles, depending in particular upon sex and to some extent upon nutritive condition. An extensive growth of comb and wattles would increase the body weight somewhat, but would have an entirely disproportionate effect upon surface area.

Using the method of least squares, the Meeh formula

$$S = kW^{.667}$$

was fitted to the data in Table I, with the result that k was evaluated at 10.64. The calculated areas of the birds by means of this are given in column 3 of Table II, and the percentage deviations from the observed values in column 4. The average percentage deviation, disregarding signs, is 4.51.

TABLE II

A COMPARISON OF CALCULATED AND OBSERVED SURFACE AREAS

Bird No.	Observed surface area sq. cms.	$S=10.64\ W^{.667}$ sq. cms.	Per cent difference	$S=8.19\ W^{.728}$ sq. cms.	Per cent difference	$\frac{W^{.333}}{L}$
8	227	244	+ 7.49	226	−0.44	.266
9	220	243	+10.45	224	+1.82	.265
11♂	376	405	+ 7.71	385	+2.39	.247
12♂	526	519	− 1.33	501	−4.75	.254
13♂	618	624	+ 0.97	608	−1.62	.264
14♂	731	718	− 1.78	707	−3.28	.249
15♂	781	738	− 5.51	727	−6.91	.249
16♀	795	813	+ 2.26	805	+1.26	.246
21♀	908	947	+ 4.30	946	+4.18	.239
18♀	1014	1052	+ 3.75	1058	+4.33	.252
23♀	1038	1105	+ 6.45	1115	+7.42	.243
17♂	1155	1114	− 3.55	1124	−2.68	.253
22♀	1127	1115	− 1.06	1126	−0.09	.230
20♀	1174	1140	− 2.90	1151	−1.96	.252
19♀	1152	1210	+ 5.03	1227	+6.51	.257
5♀	1172	1249	+ 6.57	1269	+8.28	.268
7♀	1247	1286	+ 3.13	1308	+4.89	.244
26♂	1470	1368	− 6.94	1396	−5.03	.249
6♀	1469	1391	− 5.31	1421	−3.27	.249
10♀	1435	1402	− 2.30	1433	−0.14	.244
27♂	1602	1487	− 7.18	1526	−4.74	.246
24♂	1684	1573	− 6.59	1620	−3.80	.253
28♂	1612	1598	− 0.87	1646	+2.11	.255
25♂	1720	1676	− 2.56	1732	+0.70	.249
29♂	1894	1767	− 6.71	1832	−3.27	.260
Average			4.51		3.73	.251

If the exponent of W (body weight in grams) in the Meeh formula, as well as its coefficient k, are evaluated from the data by the method of least squares, the prediction formula becomes

$$S = 8.19W^{.728}.$$

The calculated areas of the birds by this formula, and the percentage deviations, are given in columns 5 and 6 of Table II. The average percentage deviation is 3.73, somewhat less than that obtained with the first formula, and the fit to the data is appreciably better at the two ends of the range. The second formula is thus a distinct improvement over the first. Of the 25 cases only 5 show deviations greater than 5 per cent, and all are within 10 per cent. Closer fits of prediction formulas to surface area measurements have been obtained with other animals, but, as already explained, the prospects of obtaining a close fit of any formula to surface area measurements in chickens are not encouraging.

An attempt was made to improve the formula by the introduction of a term defining the nutritive condition of the animal. According to Cowgill and Drabkin (2), a term that should serve this purpose is obtained by dividing the cube root of the body weight by the body length. In the last column of Table II, this factor, involving the length in centimeters from tail to tip of beak, is given for each bird. If this factor is capable of serving a useful purpose in improving a prediction formula involving only the body weight, it would be expected that, for birds whose calculated areas deviated most widely from the observed, the nutritive correction factor would also be out of line. But a comparison of the last 2 columns in Table II does not reveal such a situation. It is true that the greatest positive deviation, 8.28 per cent, is associated with the highest nutritive correction factor, 0.268, but the next highest factor, 0.266, is obtained with a bird, No. 8, for which a very close prediction of surface area was obtained; this is also true of the next two highest factors, 0.265 and 0.264. The lowest nutritive factor, 0.230, is also associated with a bird for which a very good prediction was secured. These considerations do not indicate that the cause of poor predictions by the use of the second prediction formula was a variable nutritive condition of the birds. Hence, no systematic attempt was made to introduce this factor into the prediction formula.

From the fact that the six largest positive percentage deviations of predicted from observed areas relate to females, while the five largest negative deviations relate to males, it seems evident that sex is a determining factor in surface area, even before excessive comb growth is present (Nos. 12 and 15). Unfortunately, the present data are not suitable for the derivation of separate prediction formulas for each sex, since the females measured are all of intermediate weight, while the males are, with two exceptions, either lighter than 578 grams or heavier than 1653 grams.

Summary

The surface area of Single Comb White Leghorn chickens of both sexes may be predicted by the equation

$$S = 8.19\ W^{.706}$$

S being the surface area in square centimeters and W the body weight in grams. This formula is a distinct improvement over the Meeh formula, and apparently cannot be improved by the introduction of a factor defining nutritive condition. Except for the distribution by weight of the pullets and cockerels measured in this experiment, it would be profitable to devise separate prediction formulas for each sex.

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A COMPARATIVE STUDY OF LIQUID AND DRY MILK AS ANEMIA-PRODUCING DIETS

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VARIOUS researches have directed attention to the existence of minute amounts of certain metals in the animal organism. Body tissue and fluids, and numerous dietary substances, have been subjected to analytical survey for the purpose of explaining the significance of these small amounts of metal, but with the exception of iron and possibly copper, little progress has been made in determining their physiological function. Milk as a body secretion and as a food substance has claimed the attention of numerous investigators interested in determining basic relationships between the elemental composition of this product and the complete dietary. The normal iron content of milk has been quite definitely determined and within recent years the normal copper content of milk appears to have been fairly well established (1–5). Recent investigations dealing with the causes of nutritional anemia of experimental animals fed on an exclusive milk diet have centered attention on these two elements (6–12). The general conclusions from these investigations, either stated or implied, are to the effect that the anemia produced by a prolonged milk diet is due primarily to the low iron and copper content of the milk. The specificity of copper in preventing or curing nutritional anemia is not wholly conceded, however, by Elden and collaborators (13) or by Drabkin and Waggoner (14).

The apparent importance of the iron and copper content of milk as factors determining its adaptability for certain dietaries, has brought into relief the significance of numerous observations made at this laboratory during several years of study of the factors of similarity and dissimilarity between natural fluid milk and dry milk as prepared by the revolving cylinder process. Unpublished data obtained in 1919 revealed that desiccated milk prepared by this method normally contains from 2 to 5 times more iron than the natural liquid milk. This increase in iron content is obviously due to the intimate contact of the milk with the desiccating cylinders. Attempts to segregate this additional iron increment as an inorganic substance have failed thus far. There is reason to believe that it may exist in the dry milk as an organic combination.

The desiccating operation is known to affect certain constituents of the milk, particularly the proteins. Numerous clinical observations (15) seem to indicate that this alteration has a favorable effect upon the assimilability of the product. It is conceivable, therefore, that the assimilation of any existing iron compounds in the dry milk, particularly if associated with the protein material, may likewise be favorably affected.

Other investigations, particularly those in reference to the keeping quality of dry milk (16), led to the discovery that under certain conditions minute amounts of copper existing as an impurity in the desiccating cylinders, might be added to the dry product during the desiccating operation. Analyses of the metal of the drying cylinders revealed a copper content varying between 0.032 and 0.185 per cent. Milk dried on cylinders which were relatively new leached out the soluble copper to a variable degree, causing an increase in the copper content of the dry milk (calculated to the original fluid milk basis) of from 0.04 to 0.37 parts per million. Older drying cylinders, which had been subjected to the continual solvent action of the milk daily for a period of years, did not give up measurable amounts of copper to the milk dried on them.

Studies designed to determine whether manganese was imparted to the dry milk during the desiccating operation have also been made. Analyses of the metal of the cylinders have revealed a fairly constant manganese content varying from 0.11 to 0.17 per cent. Manganese has not been found in the dry milk, either by the usual analytical methods or by spectroscopic analysis.

In addition to these various analytical results, dry milk prepared by the roller process, used for various feeding experiments, has failed to cause the degree of nutritional anemia commonly reported for natural fluid milk. These general observations, accruing over a period of several years, taken together with clinical reports which have occasionally come to our attention indicating a degree of improvement in certain anemic conditions and emaciations when this dry milk was used, have prompted a systematic and comparative study of this type of dry milk in reference to the anemia problem.

EXPERIMENTAL

Preliminary Data

Since control measures for preventing extraneous copper contamination in the milk handled at the particular factory furnishing the product for the following experiments have been in operation for several years, abundant data were available showing the normal copper content of the liquid

and dry product. These data show that the copper in the dry milk when calculated to the liquid basis is substantially the same as for natural fluid milk, namely, within the limits of 0.50 to 0.75 milligrams per liter.[1] A few comparative analyses for iron confirmed previous data showing an increase in iron content in the dry milk to three or four fold greater than that in the liquid milk from which it was prepared.

Although preliminary feeding tests were started prior to the recently published investigations indicating the importance of the copper constituent, the analytical data from the test samples permit an accurate record of both the copper and iron content of the liquid and dry milk used in the preliminary work, the plan of which follows:

A few days prior to the birth of the young rats, several mothers were isolated in individual cages with access to the usual stock ration consisting of corn meal 76 parts; linseed meal 16 parts; crude casein 5 parts; alfalfa meal 2 parts; sodium chloride one-half part and calcium carbonate one-half part. Within 4 days after the birth of the litters the stock feed and water were taken from the mothers and the milk diets supplied fresh each day thereafter. One group received fresh liquid milk. The second group received the desiccated product, reconstituted to the original fluid milk basis with distilled water, to furnish the same content of milk solids per unit of volume as was furnished in the natural fluid milk. The third group received the same desiccated milk as the second group, but in this instance the dry product was reconstituted to give a mixture containing 30 per cent solids or approximately 3 times the milk solids per unit of volume received by the other two groups.

As the young of each litter reached the weaning age they were placed in individual cages of lacquered galvanized wire cloth and continued on the respective diets received by the mothers during the suckling period. Beginning at 30 days of age the food consumption for each individual rat was recorded. At 30, 60, 90 and 120 days of age 4 to 6 rats receiving the respective diets were killed and certain analytical determinations made as shown

[1] A recent report by Elvehjem, Steenbock and Hart (17) states that the normal copper content of milk is materially lower than these figures. Their results were obtained by a method wherein the copper was precipitated as the sulphide. According to experiences in this laboratory, the precipitation of such minute amounts of copper as are found in milk by hydrogen sulphide cannot be relied upon as quantatively accurate. The results as obtained in this laboratory by hydrogen sulphide precipitation methods have been too low, as has been readily determined by appropriate check analyses and corroborative evidence from solubility tables. Various check analyses, wherein reagents, apparatus and other important checks on the manipulations, which have been made at intervals during the past 10 years have indicated the Xanthate method as suitable for determining copper in milk.

TABLE I

AVERAGE ANALYSES OF WHITE RATS MAINTAINED ON STOCK COLONY RATION, LIQUID MILK AND RECONSTITUTED DRY MILK

Ration and age at time of analysis	Weight of rat (gms)	Dry matter (gms)	% Dry matter	% Ash on dry basis	Copper on dry basis (p.p.m.)[1]	Iron on dry basis (p.p.m.)[1]	Ratio Copper to Iron (dry basis)	Blood Count (Millions)
Stock colony ration, grain mixture and whole milk *ad lib.*								
1 day	5.45	0.85	15.73	10.97	22.0	315.4	1:14.3	
10 days	10.33	2.00	19.35	11.08	16.0			
30 days	46.09	12.88	27.94	9.97	9.7	119.3	1:12.3	7.95
60 days	127.91	38.99	30.49	10.89	4.5	117.8	1:26.1	9.73
90 days	184.80	59.18	32.02	11.61	4.6	96.5	1:20.9	9.33
120 days	211.50	72.40	34.23	11.54	4.3	100.8	1:23.4	11.01
Liquid milk only								
30 days	23.89	6.10	25.52	14.97	27.6	132.7	1:4.8	7.75
60 days	46.92	12.82	27.33	17.71	11.5	68.0	1:5.9	4.55
90 days	80.91	26.75	33.06	13.09	5.8	53.2	1:9.1	7.34
120 days	73.20	20.75	28.34	19.42	11.7	69.1	1:5.9	5.93
Dry milk reconstituted to original liquid milk basis								
30 days	29.75	7.21	24.23	13.29	22.8	166.8	1:7.3	8.25
60 days	79.40	22.80	28.71	15.36	7.2	108.2	1:15.0	11.31
90 days	117.00	39.66	33.89	13.31	3.1	98.8	1:31.9	11.38
120 days	149.00	52.00	34.90	12.05	2.6	78.9	1:30.3	12.04
Dry milk reconstituted to basis of 30% solids								
30 days	39.96	10.59	26.50	12.78	21.3	82.0	1:3.8	6.50
60 days	88.16	27.00	30.62	12.99	9.6	87.0	1:9.0	12.19
90 days	128.00	40.40	31.56	13.94	5.2	93.4	1:18.0	11.16
120 days	153.00	51.92	33.93	12.86	3.0	67.6	1:22.5	8.59

[1] Parts per million.

in Table I. For the purpose of obtaining similar data to represent standard conditions prevailing in the stock colony, other groups of young animals receiving the stock ration were isolated in individual cages and analytical data were obtained at 30 day intervals as also shown in Table I. During the observation period from the 30th to the 120th day, aliquot samples of the test milks and stock ration were taken and subsequently analyzed for iron and copper. These data with the daily or period food consumption permitted calculation of the copper and iron intake for each of the 30 day intervals. These results are shown in Table II in which is also given the average amount of copper and iron retained by the body during each 30 day period.

The results given in Tables I and II show that white rats fed on the reconstituted dry milk diet exclusively, grew better than those fed natural fluid milk. All animals receiving the desiccated product were raised to maturity. Blood counts were within normal limits and the apparent physical condition of the animals was good, although weight at corresponding ages was not so great as for those animals receiving the stock ration. Among the animals receiving the natural fluid milk diet, a number of deaths resulted, particularly between the ages of 30 to 60 days. The animals receiving this diet were emaciated and anemic, as indicated by physical condition, color of skin and eyes, and according to blood counts.

It is to be noted that the animals one day old contained a larger amount of copper per unit of dry substance than at later periods of life. This confirms the observations of Bodansky (18) that copper as well as iron is relatively high in the new born animal organism. At 30 days of age there was a greater amount of copper in the bodies of the rats receiving the milk diets than in those receiving the stock ration. The iron content of the animals receiving the milk diets was lower than of those receiving the stock ration. In the case of those receiving the natural fluid milk, the iron content of equivalent weights of dry body substance after correction for difference in growth was only about 46 per cent of that contained in the bodies of the animals receiving the stock ration; those receiving the reconstituted dry milk made up to the original liquid milk basis had about 80 per cent of the iron of the animals receiving the stock ration; and those receiving the reconstituted dry milk containing 30 per cent solids had about 57 per cent of the iron of those receiving the stock ration. At this stage of development the blood count for each group was substantially the same and obvious manifestations of anemia had not developed in any of the animals receiving the milk diets. It is apparent, however, that differences in the metabolism involving iron and copper were taking place in the body when the animals were 30 days old.

[1] Parts per million.

TABLE II

AVERAGE COPPER AND IRON INTAKE AND RETENTION BY WHITE RATS MAINTAINED ON STOCK COLONY RATION, LIQUID MILK AND RECONSTITUTED DRY MILK

Ration and Observation period	Food Consumed (gms. dry basis)	Iron Intake (mgs)	Iron gained or lost (mgs)	Iron gained or lost %	Copper Intake (mgs)	Copper gained or lost (mgs)	Copper gained or lost %
Stock colony ration 30 to 60 day period...	201	6.77	3.06+	45.2+	3.06	.051+	1.66+
60 to 90 day period.....	610	11.36	2.64+	23.2+	3.20	.096+	3.00+
90 to 120 day period.....	1218	19.38	1.59+	8.2+	6.55	.040+	0.61+
Total for 30 to 120 day period.....	2029	37.51	7.29+	19.4+	12.81	.187+	1.46+
Liquid Milk only 30 to 60 day period.....	100.3	2.50	0.07+	2.8+	0.68	0.03−	
60 to 90 day period.....	188.8	4.72	0.55+	11.6+	1.28	0.008+	0.62+
90 to 120 day period.....	24.2	0.61	0.01+	1.6+	0.17	0.08+	47.0+
Total for 30 to 120 day period.....	313.3	7.83	0.63+	8.0+	2.13	0.058+	2.7+
Dry milk reconstituted to original liquid milk basis 30 to 60 day period.....	125.8	2.18	1.26+	57.8+	1.08	0.00	
60 to 90 day period.....	281.2	4.90	1.45+	29.6+	1.94	0.04−	
90 to 120 day period.....	239.0	4.16	0.91+	4.5+	2.53	0.013+	0.51+
Total for 30 to 120 day period.....	646.0	11.24	2.90+	25.8+	5.55	0.027−	
Dry milk reconstituted to basis of 30% solids. 30 to 60 day period.....	232	5.26	1.48+	28.1+	2.48	0.034+	1.36+
60 to 90 day period.....	276	6.27	1.43+	22.8+	2.95	0.049−	
90 to 120 day period.....	253	5.74	0.26−		2.71	0.055−	
Total for 30 to 120 day period.....	761	17.27	2.65+	15.3+	8.14	0.070−	

The value of these preliminary comparisons may be briefly summarized as indicating the importance of an increased amount of available iron in the milk diet as a preventive for the characteristic milk anemia in white rats. This appears to be accomplished, in part at least, by the additional iron taken up by this type of dry milk during the desiccating process. From the foregoing data it appears that the copper content of the milk used for these comparisons may be adequate, quantitatively considered, for maintaining a substantial degree of normality of blood formation in the white rat, providing other conditions are supplied. Since the milk solids consumed per rat as reconstituted dry milk were greater than the milk solids consumed in the form of natural liquid milk, a greater copper and iron intake naturally resulted. The results at least clearly indicate the significance of an increased iron content and suggest probable importance of the assimilability of the inorganic as well as the organic constituents. Miller, Forbes and Smythe (19) have already called attention to the differences in assimilability of the iron of various protein foods.

Further Comparison of the Milk Diets

In order to study further the cause of the apparent differences in anemia-producing properties of liquid milk and the desiccated milk, a more detailed plan of investigation was undertaken.

Young rats 30 days old were isolated in carefully lacquered individual cages and various milk rations, as shown by the accompanying charts, were fed for periods up to 14 weeks. Weight, blood counts, and hemoglobin, determined with the Dare Hemometer, were taken weekly. The various milks, both liquid and dry used for the comparative study were obtained in parallel daily from a commercial milk drying plant. Slight variations in inorganic composition other than those caused by the drying operation itself were therefore avoided. All reconstituted dry milk was made up to the original fluid milk basis with distilled water. For those groups which required a supplement of milk ash, the particular sample was ashed daily and fed with the milk on the following day. Charts I to XIV have been prepared from the average of the results obtained from four rats for each group receiving the different rations. Table III shows the average food consumption and copper and iron intake per rat per week.

In order to ascertain whether minute qualitative differences in the inorganic constituents of the liquid and dry milk might account for the difference in anemia-producing results of the two products, the ash of a single sample of dry milk and the ash from the liquid milk from which it was

TABLE III

AVERAGE FOOD, COPPER AND IRON INTAKE PER RAT PER WEEK DURING OBSERVATION PERIOD

Group	Food, copper and iron intake	Weeks of Observation													
		1st.	2nd.	3rd.	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th
I—Continuous feeding liquid whole milk	Milk (cc)	227	310	313	322	298	306	283	262	277	269				
	Cu (mgs)	0.14	0.19	0.20	0.21	0.19	0.19	0.18	0.16	0.17	0.17				
	Fe (mgs)	0.30	0.41	0.42	0.43	0.40	0.41	0.38	0.35	0.37	0.36				
II—Continuous feeding, reconstituted dry milk from cylinders polished daily	Milk (cc)	271	420	518	508	563	560	570	623	647	650	684	653	669	683
	Cu (mgs)	0.16	0.25	0.30	0.30	0.33	0.33	0.34	0.37	0.38	0.38	0.40	0.38	0.39	0.40
	Fe (mgs)	0.68	1.05	1.30	1.27	1.41	1.41	1.43	1.56	1.63	1.63	1.72	1.63	1.68	1.72
III—Continuous feeding, reconstituted dry milk from cylinders not polished	Milk (cc)	266	353	449	505	552	588	575	563	650	692	761	748	743	704
	Cu (mgs)	0.14	0.19	0.24	0.27	0.30	0.32	0.31	0.30	0.35	0.38	0.41	0.41	0.40	0.38
	Fe (mgs)	0.53	0.70	0.89	1.01	1.10	1.17	1.15	1.12	1.30	1.38	1.52	1.49	1.48	1.40
IV—Continuous feeding, liquid milk supplemented with liquid milk ash	Milk (cc)	313	430	492	534	486	478	432	433	495	530	565			
	Cu (mgs)	0.36	0.50	0.57	0.62	0.56	0.55	0.50	0.50	0.57	0.62	0.65			
	Fe (mgs)	0.64	0.88	1.01	1.10	1.00	0.98	0.88	0.88	1.01	1.10	1.16			
V—Continuous feeding, liquid milk supplemented with ash of dry milk from cylinders polished daily	Milk (cc)	270	423	476	513	507	516	448	640	612	658	632			
	Cu (mgs)	0.30	0.47	0.53	0.57	0.56	0.57	0.49	0.71	0.68	0.73	0.70			
	Fe (mgs)	0.83	1.29	1.46	1.56	1.54	1.56	1.37	1.96	1.87	2.01	1.90			
VI—Continuous feeding, liquid milk supplement with ash of dry milk from cylinders not polished	Milk (cc)	269	402	427	421	455	451	473	446	501	542	487			
	Cu (mgs)	0.29	0.43	0.46	0.45	0.49	0.49	0.51	0.48	0.54	0.58	0.52			
	Fe (mgs)	0.82	1.07	1.14	1.12	1.22	1.21	1.26	1.19	1.34	1.45	1.30			
VII—Fed liquid milk, subsequent change* to reconstituted dry milk from cylinders polished daily	Milk (cc)	252	412	420	430	432	460*	395	463	530	561	605	639	640	644
	Cu (mgs)	0.16	0.27	0.28	0.28	0.28	0.27*	0.23	0.27	0.31	0.33	0.36	0.38	0.38	0.38
	Fe (mgs)	0.29	0.49	0.49	0.50	0.50	1.15*	0.99	1.16	1.33	1.41	1.52	1.61	1.61	1.62

TABLE III (*continued*)

Group	Food, copper and iron intake	Weeks of Observation													
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th
VIII—Fed liquid milk, subsequent change* to reconstituted dry milk from cylinders not polished	Milk (cc)	319	465	503	490	453*	485	529	571	669	693	743	750	751	770
	Cu (mgs)	0.21	0.30	0.33	0.32	0.30*	0.26	0.29	0.31	0.36	0.38	0.40	0.41	0.41	0.42
	Fe (mgs)	0.37	0.54	0.59	0.57	0.53*	0.97	1.05	1.14	1.33	1.38	1.48	1.50	1.50	1.54
IX—Continuous feeding, liquid milk, subsequent supplement* of ash from liquid milk	Milk (cc)	304	451	482	458*	440	447	429	374	428	541	558			
	Cu (mgs)	0.20	0.30	0.32	0.52*	0.51	0.51	0.49	0.42	0.49	0.61	0.65			
	Fe (mgs)	0.36	0.53	0.56	0.92*	0.90	0.90	0.87	0.77	0.87	1.10	1.13			
X—Continuous feeding, liquid milk, subsequent supplement* of ash of dry milk from cylinders polished daily	Milk (cc)	288	451	535	529	496	487*	458	718	660	748	717	727		
	Cu (mgs)	0.19	0.30	0.35	0.35	0.33	0.32*	0.51	0.79	0.73	0.83	0.79	0.80		
	Fe (mgs)	0.34	0.53	0.63	0.62	0.58	0.57*	1.40	2.20	2.02	2.29	2.20	2.22		
XI—Continuous feeding, liquid milk boiled for 2 minutes	Milk (cc)	327	481	527	503	460	490	454	488						
	Cu (mgs)	0.19	0.27	0.30	0.29	0.26	0.28	0.26	0.28						
	Fe (mgs)	0.43	0.63	0.69	0.65	0.60	0.64	0.59	0.64						
XII—Continuous feeding, reconstituted spray process dry milk	Milk (cc)	267	448	468	398	420	437	405	421	458	489	515	524		
	Cu (mgs)	0.17	0.28	0.30	0.25	0.27	0.28	0.25	0.27	0.29	0.31	0.33	0.33		
	Fe (mgs)	0.32	0.55	0.57	0.49	0.51	0.53	0.49	0.51	0.56	0.60	0.63	0.64		
XIII—Cont. feeding, reconstituted dry milk prepared from reconstituted spray process dry milk as used for Group XII	Milk (cc)	288	580	591	637	691	736	708	717	775	783				
	Cu (mgs)	0.28	0.37	0.38	0.40	0.44	0.47	0.45	0.46	0.49	0.50				
	Fe (mgs)	1.26	2.53	2.58	2.78	3.01	3.36	3.09	3.13	3.38	3.42				
XIV—Regular stock colony ration	Grain mixture (gms)	43	62	66	61	62	58	54	67	52	59	48	46	43	
	Milk (cc)	89	107	142	126	133	158	165	166	173	165	185	180	210	
	Cu (mgs)	0.47	0.67	0.72	0.67	0.68	0.65	0.62	0.75	0.67	0.67	0.57	0.55	0.53	
	Fe (mgs)	3.44	4.93	5.26	4.87	4.97	4.68	4.37	5.38	4.24	4.76	3.94	3.78	3.49	

made were subjected to spectroscopic examination[3]. No qualitative differences could be detected by this method of examination. The ash from a composite sample of dry milk accumulated over a period of several months likewise failed to show a qualitative constitution different from that of the ash of liquid milk. The spectrograms of these samples are shown in Plate A.

Summary

1. The production of nutritional anemia in white rats fed exclusivally on a natural fluid milk diet is confirmed. A similar anemic condition also resulted from the feeding of liquid milk boiled for two minutes, and from the feeding of reconstituted spray process milk powder containing normal amounts of iron and copper.

2. Reconstituted dry milk having the same copper content as the fluid milk from which it was prepared, but with an increased iron content resulting from contact of the milk with the drying cylinders, prevented the development of the characteristic milk anemia during the observation periods herein recorded. This milk also corrected to an appreciable degree the anemic condition resulting from the prolonged natural fluid milk diet. Immediate response to the change to the dry milk diet was shown by improved physical condition of the animals, increased blood count and ascending hemoglobin content.

3. In cases where the iron content of the reconstituted milk was approximately twice the iron content of fluid milk, the blood count and hemoglobin did not reach the levels attained by the feeding of a normal stock ration. In one group where the iron content of the reconstituted milk was approximately four times that of normal milk, a normal hemoglobin level and normal blood count were maintained throughout the observation period.

4. Increasing the iron and copper content of fluid milk as inorganic additions furnished through the medium of liquid milk ash and dry milk ash did not furnish an appreciable degree of protection against anemia, nor so great a degree of protection as did the feeding of the unsupplemented reconstituted milk.

4. The results are not conclusive in showing that the copper content of the milk is always the vital factor concerned in the anemia of white rats receiving milk diets exclusively.

5. The increased quantity of iron in the desiccated milk and such other changes in its chemical structure as may concurrently result from the desiccating operation, appear to impart measurable anti-anemic properties to this type of milk.

[3] The spectroscopic examinations were made by Dr. Jacob Papish at Cornell University.

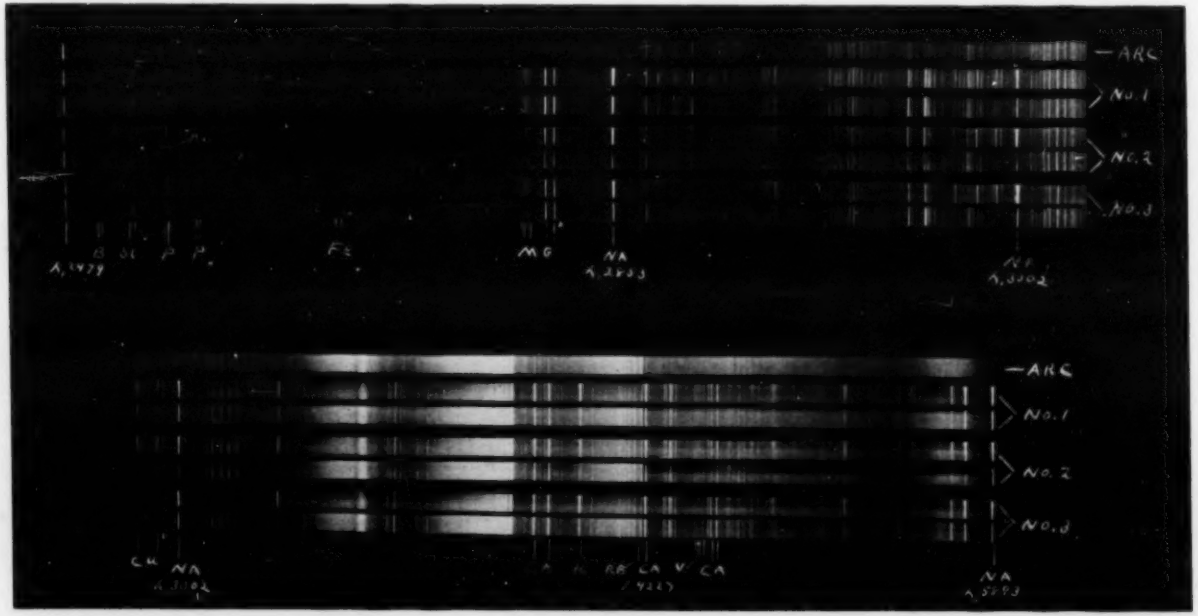

PLATE A.

SPECTROGRAMS OF LIQUID MILK ASH AND DRY MILK ASH.

No. 1. Spectrogram of a single sample of ash from roller process dry milk; No. 2, spectrogram of the ash from a composite sample of roller process dry milk; No. 3, spectrogram of the ash of liquid milk used for making the dry milk No. 1.

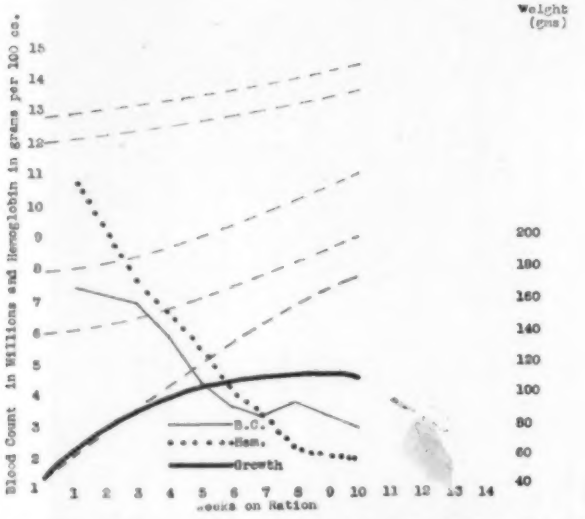

CHART 1.—Group I—Results from Continuous Feeding of Liquid Whole Milk. (Copper 0.64 p.p.m.; Iron 1.35 p.p.m.)

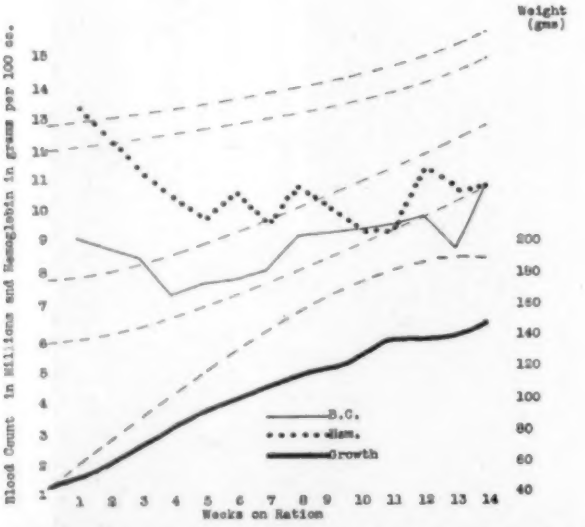

CHART 2.—Group II—Results from Continuous Feeding of Reconstituted Dry Milk Taken from Desiccating Cylinders Polished Daily. (Copper 0.595 p.p.m.; Iron 2.52 p.p.m.)

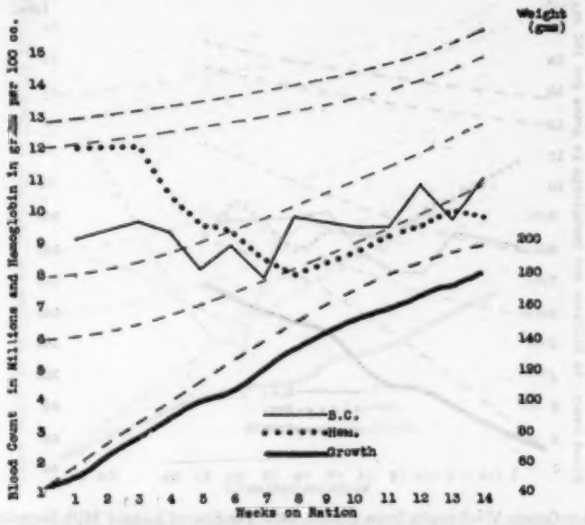

CHART 3.—Group III—Results from Continuous feeding of Reconstituted Dry Milk Taken from Desiccating Cylinders not Polished. (Copper 0.55 p.p.m.; Iron 2.00 p.p.m.)

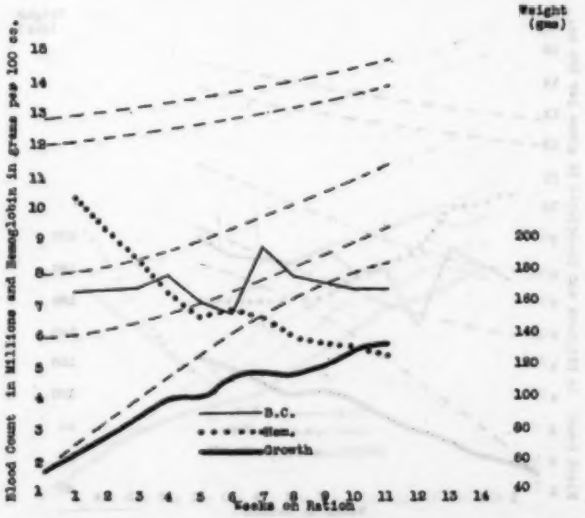

CHART 4.—Group IV—Results from Continuous Feeding of Liquid Milk Supplemented with Liquid Milk Ash—Copper in Liquid Milk Without Supplement 0.665 p.p.m.; Iron 1.18 p.p.m. (The ash from 75 cc. of the liquid milk was added to each 100 cc. of the liquid milk diet.)

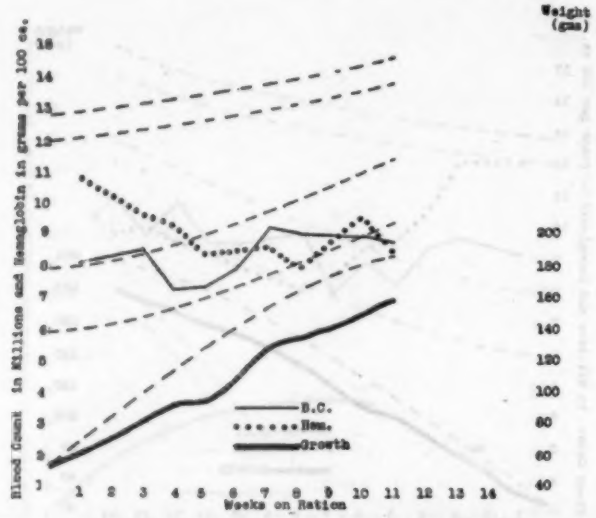

CHART 5.—Group V—Results from Continuous Feeding of Liquid Milk Supplemented with the Ash of Dry Milk Taken from Desiccating Cylinders Polished Daily—Copper in Liquid Milk Without Supplement 0.665 p.p.m.; Iron 1.18 p.p.m. (The ash from 75cc. of reconstituted dry milk—Copper 0.595 p.p.m.; Iron 2.52 p.p.m.—was added to each 100 cc. of the liquid milk diet.)

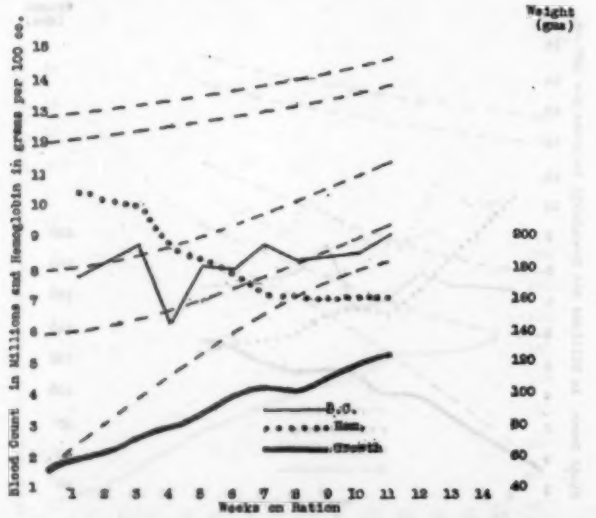

CHART 6.—Group VI—Results from Continuous Feeding of Liquid Milk Supplemented with the Ash of Dry Milk Taken from Desiccating Cylinders not Polished. Copper in Liquid Milk Without Supplement 0.665 p.p.m.; Iron 1.18 p.p.m. (The ash from 75 cc. of reconstituted dry milk—Copper 0.55 p.p.m.; Iron 2.00 p.p.m.—was added to 100 cc. of the liquid milk diet.)

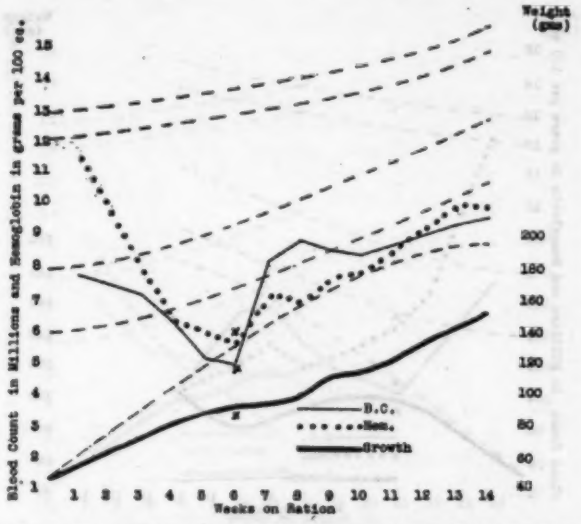

CHART 7.—Group VII—Results Showing Development of Anemia by Feeding Fluid Milk and Subsequent Improvement After Changing to Reconstituted Dry Milk Taken from Desiccating Cylinders Polished Daily. (Copper in liquid milk 0.665 p.p.m.; Iron 1.18 p.p.m.—Copper in reconstituted dry milk 0.595 p.p.m.; Iron 2.52 p.p.m.)

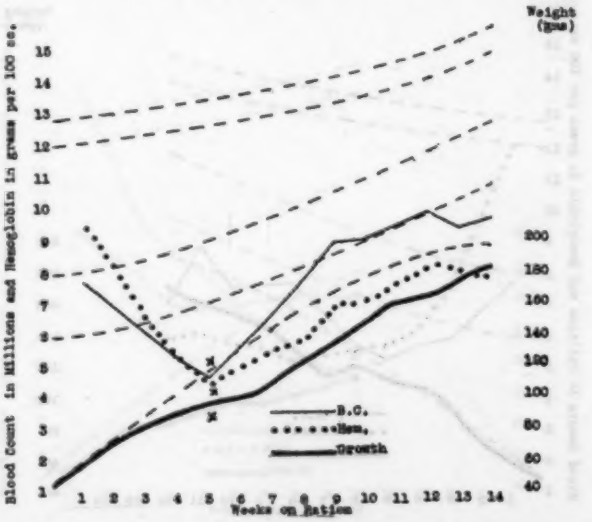

CHART 8.—Group VIII—Results Showing Development of Anemia by Feeding Fluid Milk and Subsequent Improvement after Changing to Reconstituted Dry Milk Taken from Desiccating Cylinders not Polished. (Copper in liquid milk 0.665 p.p.m.; Iron 1.18 p.p.m.—Copper in reconstituted dry milk 0.55 p.p.m.; Iron 2.00 p.p.m.)

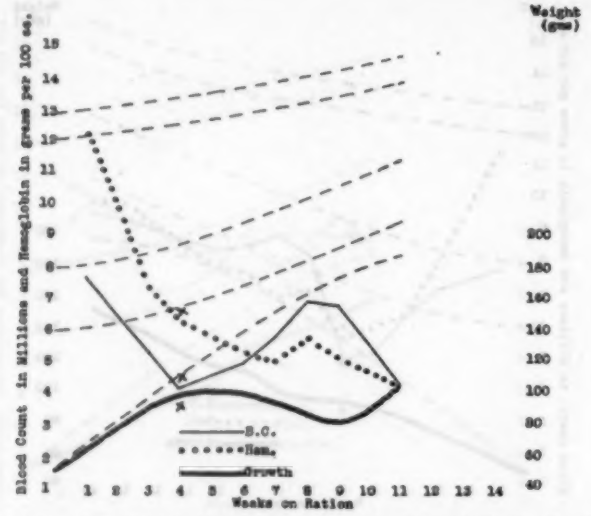

CHART 9.—Group IX—Results Showing Development of Anemia by Feeding Liquid Milk and Negligible Subsequent Effect After Supplementing with Liquid Milk Ash. Copper in Liquid Milk Without Supplement 0.665 p.p.m.; Iron 1.18 p.p.m. (The ash from 75 cc. of liquid milk—Copper 0.665 p.p.m.; Iron 1.18 p.p.m.—was added to each 100 cc. of liquid milk diet.)

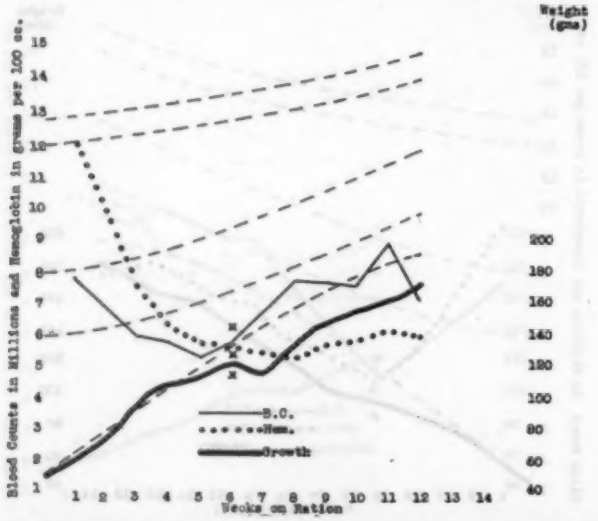

CHART 10.—Group X—Results Showing Development of Anemia by Feeding Liquid Milk and Neligible Subsequent Effect after Supplementing with Dry Milk Ash Obtained from Powder Taken from Desiccating Cylinders Polished Daily. Copper in Liquid Milk Without Supplement 0.665 p.p.m.; Iron 1.18 p.p.m. (The ash from 75 cc. of reconstituted dry milk—Copper 0.595 p.p.m.; Iron 2.52 p.p.m.—was added to 100 cc. of the liquid milk diet.)

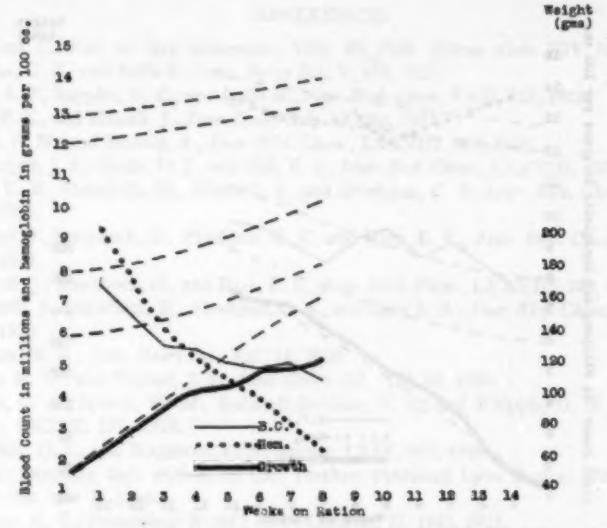

CHART 11.—Group XI—Results from Continuous Feeding of Liquid Milk Boiled for two Minutes. (Copper 0.575 p.p.m.; Iron 1.31 p.p.m.)

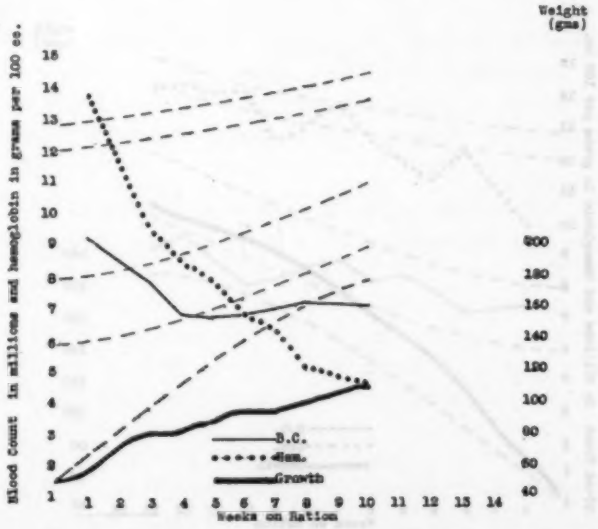

CHART 12.—Group XII—Results from Continuous Feeding of Reconstituted Spray Process Dry Milk. (Copper 0.64 p.p.m.; Iron 1.23 p.p.m.)

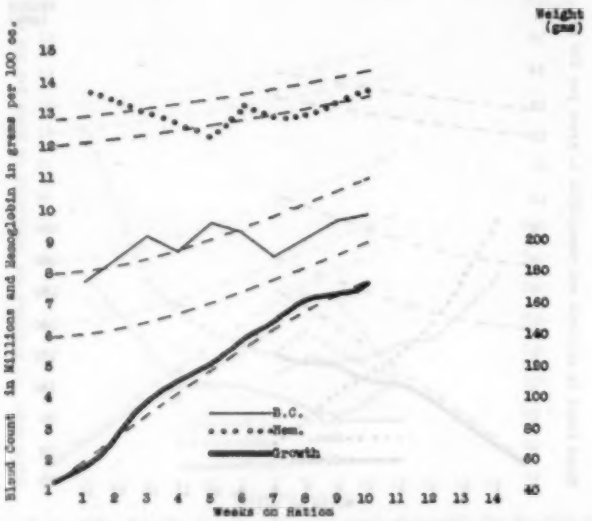

CHART 13.—Group XIII—Results from Continuous feeding of Spray Process Dry Milk Which was Reconstituted to the Liquid Basis and Again Dried by the Cylinder Process Before Feeding. (Copper 0.64 p.p.m.; Iron 4.37 p.p.m.)

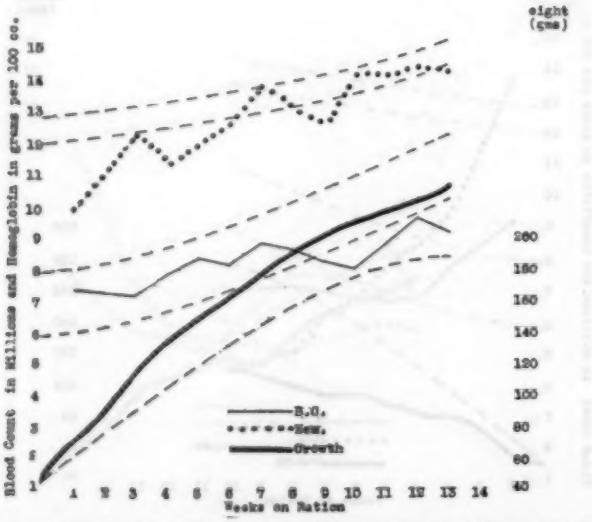

CHART 14.—Group XIV—Results from Animals Receiving the Regular Stock Ration. (Grain mixture, Copper 10 p.p.m.; Iron 77.7 p.p.m.; whole milk, Copper 0.485 p.p.m.; Iron 1.19 p.p.m.)

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THE EFFECT OF LOW CALCIUM, HIGH MAGNESIUM DIETS ON GROWTH AND METABOLISM OF CALVES

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SINCE its publication by Oscar Loew (1) the theory of calcium-magnesium antagonism has been widely accepted. It has been confirmed by Meltzer and Auer (2) and the results of many other investigators have supported it. Should it be true that the utilization of calcium is seriously impaired by a disturbance in the calcium-magnesium ratio, the inclusion in a diet of too great a proportion of magnesium-rich ingredients; *e.g.*, grain concentrates or high-magnesium mineral supplements, might produce serious results.

The evidence available does, indeed, indicate that the injection of magnesium salts (3) or their feeding (4) actually causes some decrease in calcium retention but its importance in the animal economy has been questioned, particularly if the ration supplies an abundance of calcium (5) or of phosphorus (8, 9, 10).

The purpose of the present investigation was to study the effect of magnesium when fed in connection with low calcium diets particularly after these diets had produced certain pathological conditions under which the deleterious action of the magnesium should be more marked than in normal animals.

The Effect of Feeding Magnesium Phosphate

Calves fed from birth on whole milk, or whole milk and grain, usually die in convulsions. McCandlish (6) found that calves died earlier when whole milk was supplemented with grain. He suggested that this was due to the depletion of the body stores of calcium. The fact that grains contain more magnesium than calcium causes calcium to be drawn from the body, especially the bones, to take care of the excess of magnesium. He also stated that it would appear that part of the beneficial effect of alfalfa added to a ration of milk and grain is that it increases the calcium as compared with the magnesium in the ration and thus conserves the calium stores of the body.

* Published with the permission of the Director of the Experiment Station as Journal Article No. 15 (N.S.).

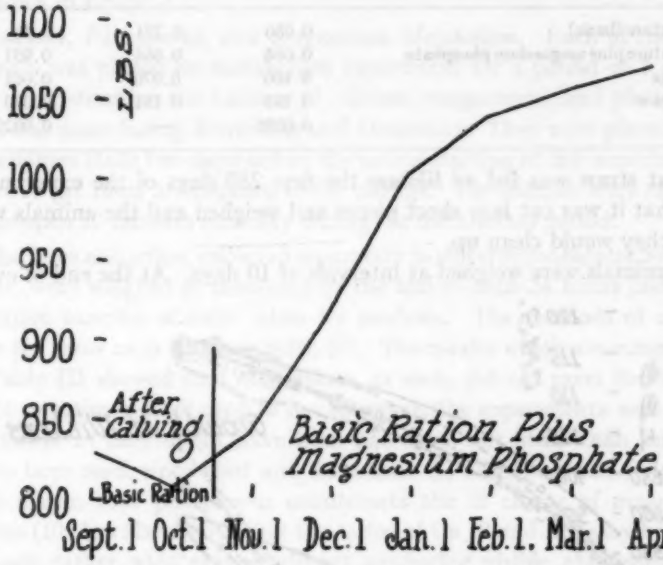
A grade Holstein calf C8 was raised on milk alone until it developed the signs of irritability which usually precede convulsions. Fifty grams of magnesium phosphate were added to the ration in an attempt to intensify the condition. However, the addition of magnesium phosphate instead of increasing irritability temporarily relieved it. The magnesium phosphate was increased whenever the animal manifested evidence of irritability until at 242 days of age 175 grams were being fed daily as shown in Table I. This animal died at 262 days of age but not in a convulsion.

TABLE I.
SHOWING GROWTH IN WEIGHT AND FOOD CONSUMPTION OF CALF C8.

10 Day Periods	Age Days	Weight lbs.	Per cent Normal	Whole Milk lbs. in 10 da.	Magnesium Phosphate gms. in 10 da.
1	12	93	90.8	76.0	—
2	22	97	86.1	92.0	—
3	32	107	86.7	102.0	—
4	42	117	87.1	120.0	—
5	52	128	86.8	120.0	—
6	62	138	86.3	120.0	—
7	72	146	83.8	134.0	—
8	82	151	80.1	140.0	—
9	92	158	77.7	126.0	—
10	102	168	85.2	150.0	—
11	112	178	75.4	176.0	50.
12	122	189	74.8	200.0	500.
13	132	203	75.3	200.0	500.
14	142	207	71.9	200.0	1000.
15	152	228	74.7	200.0	1000.
16	162	237	73.9	200.0	1000.
17	172	242	71.9	200.0	1000.
18	182	250	71.1	200.0	1000.
19	192	265	72.6	213.0	1000.
20	202	285	75.4	220.0	1000.
21	212	293	74.8	220.0	1025.
22	222	296	73.4	234.0	1300.
23	232	317	76.3	240.0	1300.
24	242	324	75.7	240.0	1750.
25	252	334	75.7	240.0	1750.
26	262	340	74.7	169.0	1225.

The following ration was fed heifer No. 205 at one year of age; 400 pounds yellow corn; 50 pounds corn gluten meal, 50 pounds cottonseed meal, 5 pounds salt, and 25 pounds wheat straw. She gave birth, after 234 days of gestation, to a blind, partially paralyzed calf which lived only two days. She also had a retained placenta.

Fifty days following parturition magnesium phosphate (which contained 0.0015 per cent calcium) was added to her basal ration at the rate of 5 per cent of the grain mixture. She was in an emaciated condition at this time. The ingested magnesium phosphate produced a slight purgative effect. There was an immediate improvement in body weight as shown in Graph 1. Her appearance also changed markedly. Unfortunately she reacted to



GRAPH 1.—Showing the gain in body weight of Cow 205 after the addition of magnesium phosphate to a ration low in calcium.

the agglutination test for *B. abortus* infection, which necessitated her removal from the experiment before calving a second time. Although her ration was very low in calcium, the addition of magnesium phosphate not only failed to aggravate her symptoms, but apparently brought about an improvement in the health and well-being of the animal.

Five grade Holstein heifers (C35, C37, C38, C39, and C46) were placed on an experiment at four months of age, up to which time they had received a recognized adequate ration. In the experiment C35, C38, and C46 were fed a basal ration consisting of wheat straw, corn silage, and the following grain ration; 3 parts yellow corn, 1 part oats, 1 part cottonseed meal, and 1 per cent salt. The other two animals, C37 and C39, received the basal ration plus 3 per cent of the grain mixture as magnesium phos-

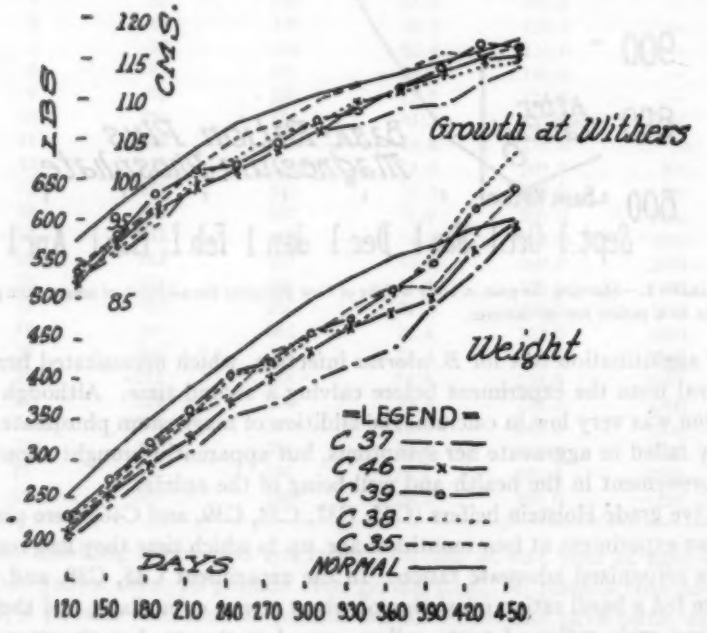
phate, a ration low in calcium and high in magnesium. The calcium, magnesium and phosphorus analyses of the feed and water fed these animals are shown in Table II.

TABLE II
SHOWING COMPOSITION OF FEEDS

	% Ca	% P	% Mg
Grain mixture (basic)	0.086	0.271	0.48
Grain mixture plus magnesium phosphate	0.086	0.664	0.951
Corn silage	0.100	0.070	0.061
Wheat straw	0.293	0.181	0.070
Water	0.0085	—	0.0029

Wheat straw was fed *ad libitum* the first 280 days of the experiment. After that it was cut into short pieces and weighed and the animals were fed all they would clean up.

The animals were weighed at intervals of 10 days. At the end of every



GRAPH 2.—Showing growth in weight and height at withers of heifers C35, C38, and C46, fed basic ration low in calcium; and heifers C37 and C39 on basic ration supplemented with magnesium phosphate.

30 day period, they were weighed 3 days in succession. Weighing was done in all cases in the morning before feeding and watering. The following measurements were taken at 30 days intervals; height at withers, height at rump, circumference of chest (just behind the shoulders), greatest circumference of barrel, depth of chest, width of chest, width of barrel, width of hooks, and width of thurls. The growth of these animals from 4 months to 15 months of age, as manifested by body weight and height at withers, is shown in Graph 2.

Calcium, Phosphorus, and Magnesium Metabolism. Each of the above animals was placed on metabolism experiment for a period of 7 days in order to determine the balance of calcium, magnesium, and phosphorus. This was done during November and December. They were placed in the metabolism stalls two days before the actual starting of the experiment in order to get them accustomed to the change. The animals were exercised in the open 20 minutes each day during the metabolism period.

The feces and urine, collected separately in suitable containers by attendants, were weighed or measured at the end of each 24 hours and representative samples of each taken for analysis. The methods of analysis were the same as in previous work (7). The results which are summarized in Table III showed that magnesium, as such, did not exert the harmful effect sometimes attributed to it. However, the experiments were not so conclusive as they might have been had some salt other than the phosphate been used, since Hart and Steenbock (8) and Haag and Palmer (9) have shown that phosphorus counteracts the ill effects of magnesium. Medes (10) has also shown that the ratios of Ca, P and Mg may be varied through rather wide ranges without producing visible abnormalities in rats unless the absolute amount of phosphorus is low. C35 and C37 were kept on the experiment for three years. During lactation, a seven-day metabolism period indicated that both animals were utilizing calcium efficiently even though C37 was receiving a high magnesium ration.

Effect of Feeding Magnesium Carbonate

A Holstein bull calf, C44, was fed a ration low in calcium but adequate in phosphorus which consisted of 2 parts ground yellow corn, 2 parts ground oats, 1 part corn distiller's grains, 4 parts corn gluten meal, 1 per cent salt and wheat straw *ad lib.* This animal became blind at about 11 months of age. At about 13 months of age the joints became enlarged and stiffness developed. This condition became very severe in June, although the animal was turned into an open lot for about 7 hours per day.

After he became so stiff that assistance was necessary to get him up from a lying position, 135 grams of commercial magnesium carbonate amounting to 5 per cent of the grain ration was added daily in an attempt to aggravate the condition. This addition was made July 7 when the animal was 467 days of age. The ingested magnesium carbonate had a purgative effect and the stools which had been firm and black became soft. An immediate improvement in health was also observed. Coincident with this, the appetite increased so that the grain fed daily was gradually increased to 9 pounds per day.

The body weight, which had remained practically constant for about four months before the addition of magnesium carbonate, gradually in-

TABLE III

SHOWING AVERAGE DAILY CALCIUM, PHOSPHORUS AND MAGNESIUM BALANCES OF ANIMALS ON A BASIC RATION LOW IN CALCIUM AND BASIC RATION SUPPLEMENTED WITH MAGNESIUM PHOSPHATE

		Animal	Feces	Urine	Milk	Total Outgo	Intake	Plus Balance	Per cent Utilized	Ca/P in food
Calcium	Period 1	C 35	5.79	0.67	—	6.45	13.47	7.02	52.1	0.71
		C 35*	8.72	2.19	21.9	32.80	41.97	9.16	74.0	0.95
		C 38	6.57	0.52	—	7.09	13.55	6.46	47.7	0.71
		C 46	3.65	0.16	—	3.81	9.06	5.26	58.0	0.55
	Period 2	C 39	5.94	0.15	—	6.09	13.63	7.55	55.4	0.40
		C 37	5.38	0.75	—	6.14	13.09	6.96	53.1	0.39
		C 37*	11.70	2.84	13.56	28.10	34.10	6.00	57.4	0.50
Phosphorus	Period 1	C 35	12.53	2.73	—	15.25	19.05	3.80	19.9	
		C 35*	18.80	4.66	15.38	38.84	44.26	5.42	47.0	
		C 38	10.19	4.31	—	14.50	19.03	4.53	23.8	
		C 46	8.03	4.74	—	12.77	16.55	3.78	22.8	
	Period 2	C 39	19.06	9.03	—	28.09	34.04	5.95	17.5	
		C 37	17.20	10.98	—	28.18	33.67	5.49	16.3	
		C 37*	39.30	4.53	10.45	54.28	68.58	14.30	36.1	
Magnesium	Period 1	C 35	14.31	1.83	—	16.13	16.70	0.57	3.4	
		C 35*	16.18	11.72	1.50	29.40	31.28	1.88	10.7	
		C 38	13.21	2.17	—	15.38	16.70	1.32	7.9	
		C 46	10.04	2.20	—	12.24	13.02	0.78	6.0	
	Period 2	C 39	23.62	3.11	—	26.73	29.21	2.48	8.5	
		C 37	22.20	2.71	—	24.90	28.60	3.70	12.9	
		C 37*	35.24	8.11	0.62	43.97	52.62	8.65	16.4	

Period No. 1, basic ration. Period No. 2, basic ration plus magnesium phosphate.

* During first lactation the basic ration consisted of 2 parts yellow corn, 2 parts linseed oil meal, 1 part oats, wheat, straw and salt.

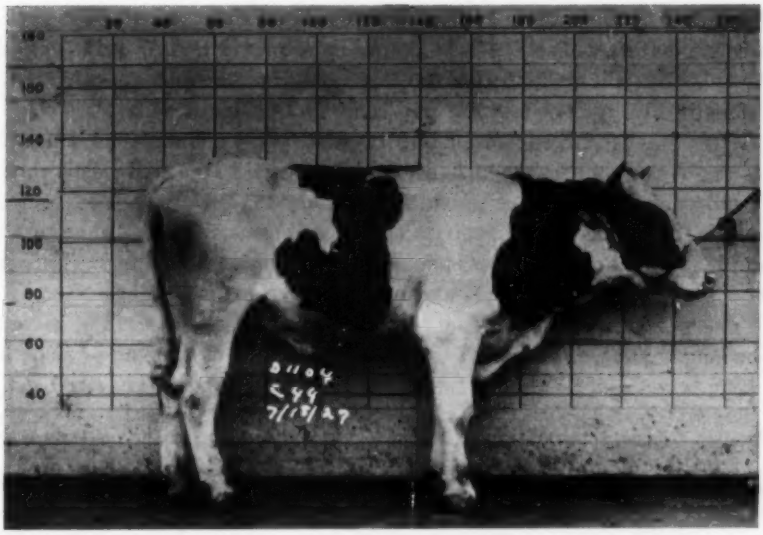


FIG. 1.—Showing animal C44 after having received commercial magnesium carbonate as a supplement to a basic ration low in calcium for one year.

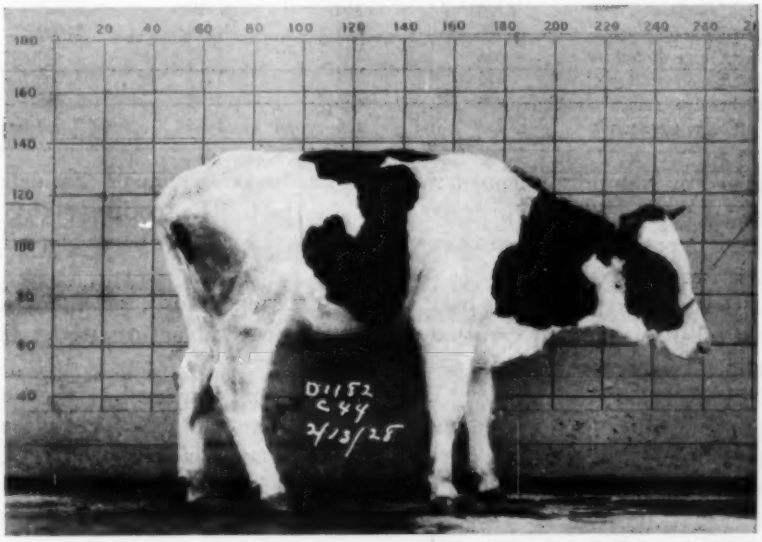


FIG. 2.—Showing animal C44 about six months after commercial maganesium carbonate had been withdrawn from the ration.

creased. The magnesium carbonate was reduced to 3 per cent of the grain mixture on September 5, at which level it was continued till July 14, 1927. At this time the animal appeared normal in health and appearance, as shown in Fig. 1. The stiffness had completely disappeared.

After magnesium carbonate had been withdrawn he appeared normal for about two months when signs of stiffness again appeared. This condition became severe in December. In January 1928, he was able to get up only with considerable difficulty. The appetite was also affected adversely and his general condition was poor (Fig. 2). He was slaughtered February 14. The rib bones were soft and easily broken near the sternal ends.

The weight, volume, specific gravity, and breaking strength of some of the long bones are shown in Table IV. The volume of the bones was deter-

TABLE IV

SHOWING WEIGHT, SPECIFIC GRAVITY, AND BREAKING STRENGTH OF SOME OF THE LONG BONES OF CALF C44

Bones	Weight gms.	Volume cc.	Specific Gravity	Breaking Strength lbs.
Right femur	2200	1900	1.16	3,125
Left femur	2350	1985	1.18	2,000
Right humerus	1775	1530	1.16	2,935
Left humerus	1705	1445	1.18	2,660
Right large metatarsis	485	373	1.30	3,420
Left large metatarsis	477	353	1.35	3,705
Right large metacarpis	435	330	1.32	2,585
Left large metacarpis	433	325	1.33	2,390

mined by water displacement method. The breaking strength was determined by breaking a 6 inch section of the shaft. The specific gravity of these bones was much less than values we have found for bones of similar size from normal animals.

Calcium and phosphorus balances were determined for a seven-day period when magnesium carbonate was being fed and again without this mineral supplement. These results are shown in Table V.

From Table V it is apparent that the magnesium furnished by the magnesium carbonate was not antagonistic to calcium and phosphorus utilization since 10.38 grams of calcium and 7.80 grams of phosphorus were stored during the magnesium carbonate period, while during the period without magnesium carbonate there was a loss of 2.55 grams of calcium and 2.15 grams of phosphorus per day.

Calcium, Magnesium and Phosphorus Balances. In order to determine

TABLE V

SHOWING AVERAGE DAILY CALCIUM AND PHOSPHORUS BALANCE OF CALF C44 ON BASIC RATION LOW IN CALCIUM AND BASIC RATION SUPPLEMENTED WITH MAGNESIUM CARBONATE

	Period	Feces	Urine	Milk	Total Outgo	Intake	Balance	Percent Utilized	Ca/P in food
Calcium	1	3.39	0.32	—	13.71	11.16	−2.55		0.64
	2	3.67	0.37	—	4.05	14.45	10.38	71.93	0.83
Phosphorus	1	17.69	1.84	—	19.53	17.38	−2.15		
	2	4.96	4.62	—	9.58	17.38	7.80	44.87	

Period No. 1, basic ration. Period No. 2, basic ration plus $MgCO_3$.

the effect on the calcium and phosphorus metabolism of feeding magnesium carbonate, three mature Holstein cows which were in the last stage of lactation were placed on a basic ration of timothy hay, a grain mixture containing 3 parts yellow corn, 1 part ground oats, 3 parts linseed oil meal, and 1 per cent salt. After a preliminary feeding period of 1 week, a 7 days' metabolism period was run, after which 150 grams of magnesium carbonate were fed as a mineral supplement per day per cow for 7 days (period 2) and the calcium, phosphorus, and magnesium balances were determined for both periods. However, in determining the average daily balances in period 2, only the last 5 days were used.

The cows were fed grain and hay and watered twice a day. The timothy hay was of good quality from the standpoint of color and purity. The college water supply, which contains 0.0085 per cent calcium, 0.0029 per cent magnesium and only a trace of phosphorus, was used. Commerical magnesium carbonate, which contained 1.37 per cent calcium and 26.05 per cent magnesium, was used. The animals had voracious appetities throughout the experiment.

The collection of excreta, and the sampling and analysis of excreta and feed were carried out as before. A composite sample of milk was taken for each 7 day period. The results are shown in Table VI.

DISCUSSION

The experiments described in this paper afforded no evidence of any bad effects from including 3 to 5 per cent magnesium salt in the ration. On the contrary, in every case the general health of the animals and, when

TABLE VI

SHOWING AVERAGE DAILY CALCIUM, PHOSPHORUS, AND MAGNESIUM BALANCES OF DAIRY COWS WITH AND WITHOUT MAGNESIUM CARBONATE AS A MINERAL SUPPLEMENT

		Animal	Feces	Urine	Milk	Total Outgo	Intake	Balance	Per cent Utilized	Ca/P in food
Calcium	Period 1	155	25.78	1.26	3.75	30.79	33.06	2.28	18.2	0.95
		143	26.12	1.63	4.93	32.68	33.66	0.98	2.9	0.97
		188	22.82	1.15	3.98	27.95	33.02	5.07	27.4	0.95
	Period 2	155	23.12	0.72	2.87	26.71	35.31	8.59	32.5	1.02
		143	26.49	1.51	4.88	32.87	35.91	3.04	22.0	1.04
		188	24.63	1.06	2.90	28.59	34.94	6.35	26.5	1.01
Phosphorus	Period 1	155	29.76	0.19	3.13	33.08	34.65	1.57	13.6	
		143	30.42	0.27	3.94	34.63	34.65	0.02	0.05	
		188	27.98	0.21	3.02	31.21	34.65	3.44	1.87	
	Period 2	155	24.91	0.22	2.40	27.53	34.65	7.11	27.4	
		143	30.07	0.21	3.86	34.14	34.65	0.51	12.6	
		188	26.70	0.34	2.18	29.22	34.65	5.43	21.9	
Magnesium	Period 1	155	15.92	4.00	0.58	20.50	24.31	3.80	18.0	
		143	12.92	5.56	0.78	19.27	24.51	5.24	24.5	
		188	15.99	3.88	0.48	20.35	24.29	3.94	18.2	
	Period 2	155	39.67	5.84	0.41	45.92	63.45	17.53	27.8	
		143	41.02	10.05	0.72	51.79	63.66	11.87	19.8	
		188	39.41	8.64	0.30	48.35	63.33	14.98	24.1	

Period No. 1, basic ration. Period No. 2, basic ration plus $MgCO_3$.

it was determined, the calcium and phosphorus retention, were unaffected or were improved.

In the case of the calf C8, which showed characteristic nervous disturbances ascribed by McCandlish to too much magnesium, the addition of magnesium phosphate gave relief instead of aggravating the symptoms. We have shown that these disturbances are accompanied by a hypocalcemia (11) which gives the present experiment a resemblance to those of Wenner (12) who reported that the continuous oral administration of magnesium lactate was an effective agency in preventing tetany in parathyroidectomized dogs. Luckhardt and his coworkers likewise prevented the onset of convlusions in thyroparathyroidectomized dogs by the administration of magnesium chloride. This experiment is complicated by the fact that the chloride probably exerted a beneficial effect through its acidogenic function (13) but the fact remains that the magnesium effect

TABLE VII

EFFECT OF MG ON BLOOD PICTURE OF A CALF WITH OSTEOMALACIA

Period	No. of Sample	Ca	P	Remarks
1925				
4/10–6/10	9	11.6	5.58	
6/10–8/10	7	10.8	6.87	
8/10–10/10	9	10.1	7.17	
10/10–12/10	21	10.4	6.28	
1926				
12/10–2/10	10	8.2	5.62	
2/10–4/10	9	8.7	3.29	Stiff
4/10–6/10	12	8.4	3.03	"
6/10–8/10	6	10.7	7.83	$MgCO_3$, 5% of ration
8/10–10/10	6	10.6	6.68	" 3% "
10/10–12/10	9	10.3	7.68	" " "
1927				
12/10–2/10	7	10.6	6.26	" " "
2/10–4/10	9	9.7	6.87	" " "
4/10–6/10	9	9.7	7.55	" " "
6/10–7/13	5	9.2	7.34	$MgCO_3$ discontinued
7/20–10/10	11	8.0	6.24	
10/10–12/10	9	9.3	3.62	Stiff
1928				
12/10–2/10	9	8.9	3.13	Stiff

was not potent enough to change the results which might have been expected from those produced by the analogous calcium salt.

The addition of magnesium phosphate to the ration of No. 205, which was low in calcium and fairly high in phosphorus, resulted in a marked improvement in body weight and health. Although she showed no rachitic symptoms, her physical condition and reproductive failure are evidence of the inadequacy of the ration. Her response to magnesium feeding was comparable to that of C44 which did show rachitic symptoms.

The case of C44 is so unique in many respects that it deserves special attention. In the first place, an animal on a low calcium, high phosphorus, diet should develop a condition characterized by low blood calcium rather than low blood phosphorus. The picture actually produced is identical with the rachitic condition[1] described by Karelitz and Shohl (14) for rats and Shohl and Bennet (15) for dogs on a *high calcium, low phosphorus* diet.

[1] Whether or not our subject developed a true case of rickets we are unable to state because of insufficient histological data, but the symptoms and blood picture were typical and the gross appearance of the bones showed skeletal abnormalities resembling rickets.

The addition of 3 to 5 per cent of $MgCO_3$ to the diet produced a clear-cut improvement in the condition of the animal coincident with the alteration of the blood picture. This is directly contradictory to the findings of Park and his associates who were able to produce typical low-phosphorus rickets "by the addition to the diet of magnesium carbonate in quantities varying between 1 and 4 per cent" (16). They are also at variance with the recently reported results of Elmslie and Steenbock (17) who, though finding no positive deleterious action with magnesium carbonate, found no curative action.

Two factors must be considered in connection with this animal, the difference in calcium intake and the laxative action of the magnesium carbonate.

During the period when the magnesium was being fed, the calcium intake was 14.45 grams per day of which 10.38 grams were stored. When magnesium was discontinued, the calcium intake was reduced to 11.16 grams. Simultaneously the storage dropped to −2.55 grams and the animal became stiff. The discrepancy in calcium storage in the two cases indictates that it was not the slight reduction in calcium intake that was responsible for the disturbance produced by the withdrawal of the magnesium. This position is supported by the fact that the phosphorus storage also fell from 7.80 to −2.15 grams per day. The importance of the second factor cannot be estimated but it should have been as active in the experiments of the other investigators cited above as in ours. This experiment is being repeated with several animals.

While the metabolism results with the above animals showed more pronounced beneficial results from the feeding of magnesium than did the others, none of the latter could be interpreted as indicating a harmful action by this material as no increase in the loss of calcium or phosphorus accompanied its inclusion in the ration.

With the other animals the influence of the magnesium compounds on the paths of excretion was not marked, but in the case of C44 magnesium carbonate reduced the loss of calcium and phosphorus in the feces and, to a smaller extent, increased their excretion in the urine.

The magnesium from the carbonate was mostly eliminated through the bowel though there was a slight increase in urinary magnesium. Either carbonate or phosphate increases the storage of magnesium though the former is apparently more effective in this respect.

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DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

XXII. FURTHER STUDIES OF THE ROLE OF MILK FAT IN FERTILITY AND LACTATION*

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IN ORDER to secure more evidence of the effect of the increase of small amounts of butter fat in a ration satisfactory for growth, but not entirely adequate for lactation (1), a series of experiments was initiated during October of 1926, which was continued for five generations. This investigation was completed during March of 1929.

The results of the experiments are summarized in Tables I to III. It will be noted that rations 995, 996, and 997 vary essentially only by 2 per cent of butter fat, the differences in whole wheat and skimmed milk powder being negligible from the standpoint of reducing the proteins and vitamin E. Since, however, dry skimmed milk contains 1.74 per cent residual milk fat left after skimming (2), the total milk fat of rations 995, 996 and 997 would be 5.56, 7.56, and 9.52 per cent respectively. The total milk fat of rations 998, and 999, containing whole milk powder is 9.17, or 8.60 per cent butter fat, and 0.57 per cent residual milk fat (3). The purpose of introducing rations 998 and 999 (999 is a duplicate of 998) was to compare the biological value of the fat-soluble vitamins in milk fat carried by whole milk powder with the fat-soluble vitamin content of skimmed milk powder

Table I

Percentage Composition of Rations

Ration	Whole wheat	Skimmed milk powder	Whole milk powder	Butter fat	NaCl
995*	61.7	32.0	—	5.0	1.3
996*	59.7	32.0	—	7.0	1.3
997*	59.7	30.0	—	9.0	1.3
998*	66.7	—	32.0	—	—
999	Duplicate of Ration 998				

* At mating the ration of the fifth generation animals was changed so that two per cent wheat oil replaced an equivalent amount of dextrin.

* Research paper No. 141, Journal Series, University of Arkansas.

TABLE II

FERTILITY AND LACTATION RECORD OF ANIMALS ON RATIONS, COMPOSITION OF WHICH IS GIVEN IN TABLE I

Ration	Generation	Reproduction period (days)	Females	Litters	Young born	Young born alive	Young allowed to be reared	Young Weaned		Remarks
								No.	per cent	
995	1st	286	3	20	175	160	110	45	41	
	2nd	113	4	4	35	35	24	15	62	One female was sterile.
	3rd	158	3	6	57	57	36	36	100	One female was sterile.
	4th	267	3	12	85	82	63	24	36	
	5th	143	5	4	28	28	24	0	0	One female died 73 days after mating, and two were sterile.
996	1st	301	3	18	146	144	105	76	72	
	2nd	218	4	8	46	42	40	15	37	
	3rd	153	2	6	43	41	34	12	35	
	4th	244	3	3	20	20	17	17	100	One female developed tumor, was eliminated before mating.
	5th	170	4	2	8	8	8	0	0	One female, sterile, died 60 days after mating.
997	1st	311	3	18	150	150	105	102	97	
	2nd	235	4	17	144	141	95	57	60	
	3rd	154	6	12	85	73	61	33	54	Two females were sterile.
	4th	341	3	12	73	68	56	42	75	One female, one male died three months before completion of experiment.
	5th	187	4	9	49	41	40	21	52	

TABLE II (*continued*)

998	1st	285	2	85	85	53	41	77	
	2nd	229	4	94	92	62	40	64	
	3rd	162	4	78	77	67	33	49	
	4th	244	4	45	36	32	22	69	Two females completely failed in growth 60 days before termination of experiment. One of these died.
	5th	151	3	19	13	13	6	46	
999	1st	285	3	147	147	100	87	87	
	2nd	235	5	130	118	117	51	44	
	3rd	159	4	44	19	19	9	47	
	4th	77	4	11	11	10	5	50	Two females sterile. Out of five young reared, only one was female.
	5th	49	1	0	0	0	0	0	

supplemented by butter fat, as evidenced in studies of lactation conducted during a period of several years. An examination of Tables II and III shows that for every increase of 2 per cent of butter fat there is a corresponding increase in 12 per cent of young successfully weaned. It is also quite apparent that ration 997 produced optimum results in both fertility and lactation. The greater number of young born on ration 995 than on ration 996 I am unable to explain. The higher percentage of sterility and dead young born on ration 995 compared with ration 996, however, is evident.

On neither ration 995 nor 996 were young reared in the fifth generation and the increase of only 2 per cent butter fat changed the lactation index in the fifth generation from entire failure to 49 per cent efficiency, as indicated by the results secured on rations 996 and 997 (Table II).

It is also evident that rations 998 and 999, which are duplicates of Sherman and Muhlfeld's whole milk powder diet B (4), containing a total of 9.17 per cent milk fat, did not prove as efficient as ration 997, containing only about 0.4 per cent additional milk fat. Table III shows that the increase of lactation efficiency on rations 996 and 997, compared with results secured on ration 995, follows a curve of arithmetical progression, *i.e.*, for every 2 per cent additional amount of butter fat there is a corresponding increase of 12 per cent in rearing of young; therefore, 0.4 per cent less butter fat should have produced a 2.4 per cent less efficient lactation record

TABLE III

SUMMARY OF FERTILITY AND LACTATION RECORDS OF ANIMALS ON RATIONS 995 TO 999 INCLUSIVE COVERING FIVE GENERATIONS

Ration	Total reproduction period (days)	Females	Sterile females	Litters	Young born	Young born alive	Young allowed to be reared	Young weaned	
								No.	per cent
995	967	18	4	46	380	362	257	120	47
996	1,086	16	1	37	263	255	204	120	59
997	1,228	20	2	68	501	473	357	255	71
998	1,071	17	0	44	321	303	227	142	62
999	805	17	2	44	332	295	236	152	64

on rations 998 and 999 than on ration 997. Actually, there was a decrease of 9 per cent, or for every 65.4 young expected to be weaned out of 100 there was a loss of 5.6 per cent efficiency. This slight difference in lactation efficiency, however, may not at all be due to destruction of vitamin A in whole milk powder during the process of drying, but may be due to biochemical changes produced in the whole milk powder during storage of that material in summer months in our laboratory.

An examination of all the milk powder diets used in this investigation reveals that none can be considered optimum for lactation. The fortification of the rations in the fifth generation with vitamin E furnished by wheat oil did not improve the lactation efficiency index. On the other hand in a good many instances we have observed polyneuritis in failing nursing young which readily responded to vitamin B therapy, the vitamin being furnished to the nurslings in the form of concentrated extracts from yeast or rice polishings. It is, therefore, concluded that vitamin B is one of the limiting factors in such milk diets. That milk is deficient in vitamin B for lactation was demonstrated in 1924 (5), since rations composed of 50 per cent skimmed milk powder had to be supplemented with a brewer's yeast concentrate, in order to make them adequate for rearing of young. The irregular and conflicting results recently reported by Daniels, Jordan and Hutton (6) on the potency of milk in vitamin B for lactation are due to the failure of these investigators to deplete the lactating mothers of vitamin reserves, and also to the failure to use an experimental maternal diet satisfactory in every respect with the exception of vitamin B. For the same reasons the conclusions of Daniels, Giddings and Jordan (7) on the destruction of vitamin B in evaporated milk also are open to criticism.

Although we find the Sherman and Muhlfeld diet B (our rations Nos.

998 and 999) unsuitable for raising a vigorous rat colony, which conclusion is in agreement with the recent report of Smith and Anderson (8) we consider that diet, supplemented with fresh vegetables, preferable for raising animals preparatory to studies on vitamins A or D, since it allows less storage of such fat-soluble vitamins.

Summary

1. The importance of small variations in the amount of milk fat in fertility, and particularly in lactation, is demonstrated.

2. One of the limiting factors in all the milk diets studied from the standpoint of lactation is vitamin B.

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THE COMPARATIVE VALUE OF DIFFERENT FOOD PROTEINS FOR REPRODUCTION AND LACTATION IN THE RAT*

I. BEEF MUSCLE, LIVER AND KIDNEY

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THE science of nutrition has been greatly advanced during the last two decades through an increase in knowledge of the unequal nutritional values of amino acids, the rôle of mineral salts in vital processes, and the indispensability of vitamins. The biological method of experimentation was applied to the study of foods at first only in so far as they concerned growth and maintenance. It was McCollum (1915) who first suggested the importance of such study for reproduction and lactation. "Only when normal reproduction and rearing of young is repeated at normal intervals can a ration be said to be physiologically sufficient." Since that time much effort in this direction has revealed that for the proper performance of these functions an animal has very definite qualitative and quantitative needs in the way of dietary constituents. These needs, as far as they were known, were stated clearly by Simmonds (1924). If these needs are not met, young may never be born, or if they are born, their lives may be brief because of the lack of some necessary constituents for the formation of normal milk. Sometimes the young are normal when born and remain so until weaned, but only at the expense of the mother who is found in an emaciated condition, due to the vicarious supply from her own tissues of certain substances indispensable for milk production. Or, finally, the young may be born and reared normally but prove to be incapable of procreation.

The acquisition of data is made the more difficult by the larger number of variables involved, most of them of unknown magnitude. Before a qualitative or quantitative inadequacy in one dietary constituent can be accepted as proved, the "law of the minimum" for all other dietary requisites must first be complied with. A recognition of the indispensable food entities has been and is being achieved only by the same biological

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method, and it is therefore not surprising that often purely accidental variations in diet have led to the discovery of hitherto unsuspected essentials. Vitamin E came into being as the proper interpretation of the results of a chance dietary variation (Evans, 1922, 1923).

The need of increased amounts of vitamins for reproduction and still larger amounts for lactation over the maintenance requirements has been demonstrated (Sure, 1926, 1928). It is well known that a protein of good quality is necessary for normal reproduction and lactation, but the quantity required has not been exactly determined. McCollum (1915) first emphasized the importance of mineral salts for normal procreation. Many workers in this field have attempted to supply all of the presumably necessary elements but much remains to be learned about the actual salt requirements with different kinds of diets, the actual needs of the body for certain of the rarer elements, and the catalytic, or other, effects which certain of them, such as iron and copper, have upon the body processes.

The purpose of the experiments described in this and the following paper was to compare beef muscle, liver, kidney, milk and egg for reproduction and lactation in the rat.

HISTORICAL

The use of an exclusive diet of muscle or gland tissue has always been followed by nutritive failure. Of C. Watson's (1906) 14 young rats on ox flesh, 5 died within four months, the others were underaized and sterile. Partly grown rats on this food became pregnant but failed to rear their young. The similar experiments of B. P. Watson (1907) in which partly grown rats were subjected to short periods of meat feedings, resulted in poorly nourished litters and subnormal weights of the mammae of the mothers.

Later work on muscle tissue by McCollum (1921) and Osborne and Mendel (1917, 1918) revealed that this is low in vitamin B and lacking in vitamin A and calcium. When fed on a 20 per cent level with mineral and vitamin A supplements, animals did not attain normal growth, and reproduction and lactation were unsuccessful. This suggests an inferior quality of protein and also inadequate vitamin E. The contrary observations of Evans and Burr (1927) who, to be sure, used a different technique, are illustrative of the influence of unknown variables.

Liver tissue, according to the same groups of workers, contains considerable amounts of vitamins A and B, but lacks calcium. The protein appears to be of better quality than that in muscle tissue. Kidney tissue is rather more complete. The protein is of excellent quality and the vitamin A content high. Vitamin B appears to be present in smaller amount than in liver. The content of calcium is low.

The biological values of the proteins of muscle, liver and kidney on an 8 per cent level, as determined by Mitchell (1926, 1926-1927), were 69, 77, and 77 per cent respectively. Fed on a 15 per cent level with necessary non-protein supplements, Hoagland and Snider (1926) found little difference between dried ox muscle and liver when measured by growth. The results of Hartwell (1921, 1924) were at first baffling but her conclusions were confirmed by Hassan and Drummond (1927) with special emphasis upon the need of increasing amounts of the heat-stable factor of vitamin B when protein intake is increased.

Experimental

Animals. The animals, obtained either from our own stock or from the Albino Supply Company in Philadelphia, usually weighed from 45 to 65 grams when put on experiment, seldom more. Individual charts were made for each animal. These contained the reproductive history as well as the growth curve. Females were weighed regularly once a week, pregnant animals every day. Litters were weighed once a week and males once in two weeks. The behavior of each litter was carefully noted from birth until death or weaning time. This involved daily observation of each mother and her young. During the course of the whole experiment, charts were made for 648 animals and records were kept of 711 litters.

Rations. In the first comparative rations it was thought best to supply known sources of all the food constituents which were considered to be necessary for reproduction and lactation in the rat. The protein was supplied entirely by the meat, with the exception of the small amount present in the yeast which was used as a source of vitamin B. The meat was also the only source of vitamin E. Fat, when needed, was supplied by lard, carbohydrate by cornstarch, and vitamins A and D by cod liver oil. The salt mixture used was that of Osborne and Mendel (1917). The protein and vitamin E in the meats thus became the only known variables.

In the earlier series of experiments, the meats were fed in cooked, dried form. The beef round, liver, and kidney were prepared as follows: After the removal of all visible fat they were cooked in steam in an autoclave at 10 pounds pressure for one hour. They were then ground in a meat grinder. The juices collected in the cooking vessels were evaporated to a small volume on a water bath and added to the meat which had been partially dried at a low temperature (not over 40 degrees C). The drying was completed at the same temperature and the meats were then ground to a fine powder.

The gross chemical composition of the dried meats was determined by analyses of several lots and was as follows:

	Protein	Fat	Ash
Beef round	74.01	11.40	3.69
Beef liver	62.00	10.25	4.71
Beef kidney	70.00	11.66	5.12

Series I—Moderate Protein, High Fat

The first series of rations was planned to contain 15 per cent protein, 20 per cent fat, and 5 per cent ash and on the basis of the above analysis they were constituted as follows:

COMPOSITION OF RATIONS IN SERIES I
(15% protein, 20% fat, 5% ash)

Ration No.	Product (powdered)		Starch	Lard	Salts	Yeast	C.l.o.
120	Round	20.3	52.5	15.7	4.5	5.0	2.0
130	Liver	24.2	49.3	15.5	4.0	5.0	2.0
140	Kidney	21.4	52.1	15.5	4.0	5.0	2.0

The 15 per cent protein level was chosen for a beginning because this was the lowest level at which McCollum (1921) obtained successful results in reproduction and lactation on meat diets. His diets differed from ours, however, in several respects, the chief difference being that they contained 3 per cent of butterfat, no yeast, and no lard.

In preparing the diets of series I, all of the ingredients were thoroughly mixed together in 1 or 2 kilogram lots and placed in open tin cans. As long as the food lasted it was exposed to the temperature and air of the rat room. As a rule, however, fresh food was made up once a week or oftener. No records were kept of food consumption. Food was always available to the animals.

Beef Round, Ration 120. The 8 females and 6 males on this ration grew normally, or better, without exception, but reproduction was practically a failure. Up to 125 days, only two females had borne litters. Soon after this 3 per cent of wheat germ oil was added to the ration as a source of vitamin E. All of the females then had litters but only 3 had any success in lactation. One female with lung disease was anesthetized; the other 4 were given an alcoholic extract of wheat enbryo[1] to see whether lactation would be improved. It was improved in three of them. The results were as follows:

	No. Born	No. Lived	% Lived
After oil	68	13	19.1
After oil & extract	39	25	64.0

The one female which did not show improved lactation was found to have badly diseased lungs.

The 6 males all received the 3 per cent oil addition. Only one appeared to be infertile and it showed testes weights 49 per cent of normal.

Ten second generation females and 2 males were kept on the ration with the oil addition. Their growth was normal or above. However, only 8 females had litters and only 3 had any success in lactation. There were a great many cases of diseased lungs. The males were both fertile.

Beef Liver, Ration 130. Two lots of animals were placed on this ration, and as the results differed in some respects, the two groups will be considered separately.

In the first group of 5 females and 4 males growth was normal or better and in the beginning all animals were fertile. After 175 days, however, fertility ceased, showing a deficiency of vitamin E. There was some success in lactation, 39 per cent of the young being weaned, but some were underweight.

[1] The extract was made as follows: Two liters of 70 per cent alcohol were added to 400 grams of ether-extracted wheat embryo in a Florence flask. The flask was stoppered and thoroughly shaken several times a day for five days. The extract was then filtered off with suction and evaporated to about one-half its volume by use of an electric fan at room temperature. One-half the total volume was used in one kilo of ration, the extract being poured on starch and allowed to evaporate to dryness before the other ingredients were added. This extract was used as an additional source of the vitamin B complex, or perhaps of a new accessory of water-soluble nature, mentioned by Evans, (1924).

At autopsy the parents showed considerable lung disease and a peculiar form of fatty degeneration of the liver. The tissue had a yellow mottled appearance and was tough and fibrous. Three of the males showed degeneration of the testes. The second generation animals were all sterile but showed no apparent liver changes.

The second group of animals showed better growth than the first and fertility was maintained a little longer, but lactation was not so successful, only 8 per cent of the young surviving. At autopsy the animals showed considerable lung disease but the abnormal liver condition was not found. One male and one female of the second generation were kept on the ration and both were sterile.

Beef Kidney, Ration 140. The 8 females and 5 males on this ration showed a growth rate which was normal or above. At the end of 125 days only 4 of the females had reproduced and none of the young had been raised. Since there was an apparent deficiency of vitamin E, 2 per cent of wheat germ oil was added to the ration. Five of the females then had litters but 2 remained sterile and another became so. The reason for this failure to respond to wheat germ oil was probably the fact that the females were mated with a male which was later found to be sterile. One of them was also found to have ovarian disease. There was some success in lactation following the oil addition, 41.7 per cent of the young being weaned.

Eight second generation females and 4 males were kept on the ration with the 2 per cent oil addition. All but one female grew very well but reproduction was not uniformly successful. Three females were sterile but this may have been due to the fact that they were mated with males whose fertility was questionable.

Lactation was a complete failure in two animals but fair in others, 25.7 per cent of the young born surviving and making unusually good growth.

Three females and one male from the third generation were kept on the same ration but all were sterile.

Discussion of Rations in Series I

Of the meat rations, that containing liver was easily the best so far as reproduction was concerned, but even here it failed before the end of the first generation. Since the addition of wheat germ oil greatly improved the reproductive efficiency of the muscle and kidney rations, it is reasonable to conclude that vitamin E was the limiting factor in the liver ration also. Practically the same amount of lard was present in each of the three rations, hence the superiority of liver must have been due to some quality of the liver itself. The influence of lard will be discussed later.

None of the rations in this series was very successful for lactation. Liver at first appeared to be superior, as 39 per cent of the young of the first group were weaned. In the second group, however, only 8 per cent lived.

The addition of the alcoholic extract of ether-extracted wheat germ apparently improved lactation on the muscle diet and from the work of Evans and Burr (1928) and Sure (1927, 1928) it seems probable that the effective substance in the extract was vitamin B, particularly the heat-labile fraction, which was described by Smith and Hendrick (1926), Goldberger (1926), and others and which eventually will probably be called vitamin B or B_1.[2] Wheat embryo has been found by Chick and Roscoe

[2] Following the recommendation of the Committee on Vitamin B terminology appointed by the American Society of Biological Chemists (*Science*, 1929, LXIX, 276).

(1927) to be rich in this heat-labile factor and poor in the heat-stable factor (G or B_2). Yeast contains both factors, but Evans and Burr (1928) have found that it requires 15 per cent of brewer's yeast to provide enough of each for lactation. Liver and kidney are known to contain more of the vitamin B complex than muscle, and it is probable that the method of preparing the meats in dry form greatly reduced the amount of vitamin B (antineuritic) in muscle so that the 5 per cent of yeast was no longer adequate. Hence the effectiveness of the wheat embryo extract. Unfortunately it was not used as a supplement to the liver and kidney rations, and these tissues as well as many other foods must be restudied in view of the composite nature of vitamin B.

Effect of Use of Special Salt Mixture in Liver Ration 130

The effectiveness of certain mineral additions to milk diets in promoting reproduction and lactation (Daniels, 1925) suggested the substitution of a special salt mixture[3] containing these minerals in the best ration of this series (liver).

Beef Liver, Ration 130S. The liver diet was not improved in any way by the special salt mixture. Six females and one male were placed on the ration. The 3 females which were first mated with this male had one litter each but the others which were mated with second generation males from ration 130 were sterile. Lactation was a failure.

Series II—High Protein, High Fat

The rations in Series II were made to contain 20 per cent protein with the idea that by increasing this constituent and at the same time the other constituents of the meats, the deficiencies apparent in Series I might be overcome. The rations were as follows:

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[3] The salt mixture, modified from Osborne and Mendel (1918) comprised the following:

	gms.		gms.
$CaCO_3$	539.2	Citric acid (dry)	406
$MgCO_3$	96.8	Iron citrate	25.36
Na_2CO_3	136.8	KI	1.4
K_2CO_3 1-1/2 H_2O	675.6	$MnSO_4H_2O$	8.7
85% H_3PO_4	284 cc.	NaF	8.5
Conc. HCl	504 cc.	Alum	4.2
Conc. H_2SO_4	21 cc.	$Na_2SiO_3H_2O$	25.0

COMPOSITION OF RATIONS IN SERIES II
(20% protein, 20% fat, 5% ash)

Ration No.	Product (powdered)		Starch	Lard	Salts	Yeast	C.l.o.
121	Round	27.0	46.6	14.9	4.5	5.0	2.0
131	Liver	32.2	42.1	14.7	4.0	5.0	2.0
141	Kidney	28.0	46.3	14.7	4.0	5.0	2.0

Beef Round, Ration 121. The 8 females and 5 males on this ration showed growth which was above normal. The females all had litters but in only one case did fertility continue after the animals were 175 days of age. Lactation was only slightly successful, 10.8 per cent of the young being reared. Two of the males were discarded early in life. Of the other 3, two were sterile and one was questionable.

Six females and 2 males were carried through the second generation. Growth continued to be excellent but reproduction was a complete failure.

It is therefore evident that the presence of enough muscle tissue to make 20 per cent protein provided for some success in reproduction and lactation in the first generation but none in the second. Vitamin E was deficient to judge from the improvement in reproduction which was obtained when wheat germ oil was added to ration 120 containing 15 per cent of muscle protein. Vitamin B was but slightly increased in ration 121.

Beef Liver, Ration 131. The seven females and 6 males placed on this ration all showed a growth considerably above normal. As in the case of the ration containing 15 per cent liver protein the animals were at first fertile. However, after the females were 175 days of age no litters were born. Lactation was improved to some extent, 50.6 per cent of the young surviving.

The ten females and 4 males of the second generation were all sterile.

Increasing the protein to 20 per cent thus apparently had little effect in improving reproduction, but caused some improvement in lactation, suggesting an increased vitamin B supply.

Beef Kidney, Ration 141. Nine females and 5 males were placed on this ration. Growth was above normal but continued reproduction was not secured. At the age of 175 days 2 per cent of wheat germ oil was added to the ration, but with little effect, as the males had already become sterile. The few litters which were born were the result of mating with colony males. There was no success in lactation before the addition of wheat germ oil, but of the ten young born afterward, five lived. These made exceptionally good growth.

No second generation animals were continued on the ration.

Increasing the protein content of this kidney ration caused a slight improvement in reproduction but none in lactation.

DISCUSSION OF RATIONS IN SERIES II

(*See Table I, page 500*)

All of the animals on the Series II rations were obviously suffering from a deficiency in vitamin E and it is unfortunate that in several instances this was supplied too late in the life of the male animals. Their sterility had become incurable, as happens after about 150 days, and sometimes earlier, according to Mattill (1924) and Evans and Burr (1927).

As a result of recent experimentation by a number of workers it seems very probable that the deficiency in vitamin E in the rations of both Series I and II may be partly accounted for by the presence of the unsaturated animal fats, lard and cod liver oil. Anderegg and Nelson (1926) dem-

onstrated that with the development of rancidity, fats have a destructive effect on vitamins which is associated with processes of oxidation. Ferrous sulphate also has a catalytic effect on oxidative processes, as was demonstrated by Simmonds, Becker, and McCollum (1927). The function of wheat germ oil in a ration is apparently that of an antioxidant as well as a source of vitamin E. Mattill (1927) thinks that a possible explanation for this protective action of wheat germ oil is found in the experiments of Holm (1927) to the effect that the OH group has a very powerful retarding action upon oxidation. It thus appears that the degree of success in reproduction on a ration may be a measure of its content of antioxidants, when such a ration also contains auto-oxidizable fats.

Since the 5 per cent increase in muscle protein was most effective in improving reproduction, it might be argued that muscle contains more antioxidant than kidney or liver, a statement which agrees with Evans' observations on the distribution of vitamin E in these same tissues.

As regards lactation, liver took first place, followed afar by muscle and kidney. The kidney ration, like that in Series I, was of no value for lactation until after the addition of 2 per cent of wheat germ oil. It would seem that dried beef kidney may contain a considerable amount of the heat-labile vitamin B. In the presence of sufficient vitamin E, this becomes available for lactation.

The protein of kidney is apparently of unusually good quality for growth. The young rats on ration 141 were of very large size at weaning time.

Series III—Medium Protein, Low Fat

With the object of further testing the deleterious effect of lard in rat diets, Series III rations were prepared. They contained, as shown below, a quantity of fat (10 per cent) more nearly like that usually present in human dietaries and slightly more than half of this was lard.

Composition of Rations in Series III
(15% protein, 10% fat, 5% ash)

Ration No.	Product (powdered)		Starch	Lard	Salts	Yeast	C.l.o.
123	Round	20.3	62.5	5.7	4.5	5.0	2.0
133	Liver	24.2	59.3	5.5	4.0	5.0	2.0
143	Kidney	21.4	62.1	5.5	4.0	5.0	2.0

Rations 123, 133, 143. It was expected that these three rations would at least equal the three corresponding rations in Series I. It is rather difficult to make a direct comparison except in the case of the liver rations, owing to the fact that additions of wheat germ oil were made so early in the career of the animals on rations 120 (round) and 140 (kidney). It is possible, however, to compare the results of the two series up to the time the animals were 150 days of age, as follows:

Ration		No. born per Female	Per cent which lived
Round	120	1.4	0
	123	4.4	0
Liver	130 1st lot	6.0	50
	130 2nd lot	9.3	10.7
	133	2.4	0
Kidney	140	2.3	0
	143	4.6	0

There was a slight improvement in reproduction on round and kidney following the decrease in fat. Results on liver were not so good and there seems to be no way of explaining this fact. Lactation was a complete failure in the case of all three rations. One may conclude therefore that in a ration containing 15 per cent of protein and 10 per cent of fat, part in the form of lard and cod liver oil, neither beef round, nor liver nor kidney is adequate for reproduction and lactation.

Series IV—High Protein, No Added Lard

The results secured in the rations of the first three series naturally led to a fourth containing 20 per cent of protein and no added fat except 2 per cent of cod liver oil to supply vitamins A and D.

Composition of Rations in Series IV
(20% protein, no lard, 5% ash)

Ration No.	Product (powdered)		Starch	Salts	Yeast	C.l.o.*
124	Round	27.0	61.5	4.5	5.0	2.0
134	Liver	32.2	56.8	4.0	5.0	2.0
144	Kidney	28.0	61.0	4.0	5.0	2.0

* Added daily after 135 days.

Beef Round, Ration 124. The 8 females and 3 males on the ration all showed growth which was normal or above. Before the cod liver oil was added daily, only 4 of the females had litters. Afterward all were fertile. Of the young born, 16.5 per cent lived. The 3 males all remained fertile.

Five second generation females and 2 males were kept on the ration. Growth was normal or above and fertility continued. None of the 3 females had litters up to the age of 125 days, but all were fertile later. Lactation was improved, 51 per cent of the young born now surviving.

Three females and one male from the third generation were kept on the ration. Their growth was normal, but owing to shortness of time they were anesthetized at 155 days of age, before reproduction had begun.

Beef Liver, Ration 134. Most of the 9 females and 3 males on this ration showed growth which was above normal. Two of the females were infertile and one died at the birthof her first litter before the cod liver oil was added daily. Afterward, the 8 living females all had litters. One, which showed poor fertility, had an infected uterus. Sixteen and one-tenth per cent of the young born were weaned. The males all remained fertile.

Six second-generation females and three males were kept on the ration. Growth was not quite so good and only three females were fertile. Of the young born, 27.7 per cent were weaned. At autopsy two of those which were infertile showed no scars on the uterus, proving that they had never been pregnant. Two of the females also showed slight indication of a fatty degeneration of the liver, similar to that which was noted in the first group of animals on ration 130.

Beef Kidney, Ration 144. Growth on this ration was above normal in most cases. Three of the 8 females were sterile before the cod liver oil was added daily, but afterward all were fertile. Reproduction was not so good, however, as on rations 124 and 134, and only 6.8 per cent of the young born were weaned. The animals showed considerable lung disease at autopsy, which may partly account for the poor results. The 3 males were all fertile.

Five females and 2 males from the second generation were kept on the ration. Only one female was fertile but embryos were present in 3 others at autopsy. The one fertile female raised one litter of seven and lost one. The males were both fertile.

One male and one female were kept from the third generation. The female was anesthetized at the age of 87 days but showed 9 half-grown embryos.

Discussion of Rations in Series IV

The marked improvement in reproduction following the removal of lard from the rations and the addition of the cod liver oil daily is plainly shown in Table I, in which the results on the rations of Series II and IV

Table I
Comparison of Results in Series II and IV

Ration	No. females	Av. age at birth first litter days	Av. No. lit. per female to 255 days	Av. No. born per female	Per cent that lived	Remarks
121, Round, lard +c.l.o	8	112	2.0	11.5	10.8	2nd generation sterile
124, Round, no lard, c.l.o after 135 days	8	121	2.9	23.0	11.8	2nd generation fertile
121, Liver, lard+c.l.o	7	100	1.9	11.3	50.6	2nd generation sterile
134, Li ver, no lard, c.l.o daily after 135 days	9	117	2.9	19.1	12.2	2nd generation 50% fertile
141, Kidney, lard+c.l.o.	8	121	1.4	3.1	0.0	No 2nd generation
144, Kidney, no lard, c.l.o. daily after 135 days	8	149	2.3	14.5	7.7	2nd generation partly fertile

are compared. The contrast in results in the second generation is particularly striking. Lactation was also improved in the Series IV rations containing round and kidney but not in the one containing liver.

Series V—High Protein, Test for Vitamin B

The rations of Series V were designed to indicate the relative vitamin B content of the dry meats used, and since muscle is already known to be a poor source of vitamin B (Osborne, 1917, 1918) it was omitted from the series, whose composition was the following:

COMPOSITION OF RATIONS IN SERIES V
(20% protein, no lard, no yeast, 5% ash)

Ration No.	Product (powdered)	Starch	Salts	C.l.o. *
135	Liver 32.2	61.8	4.0	2.0
145	Kidney 28.0	66.0	4.0	2.0

* Added daily after 125 days

Beef Liver, Ration 135. The 8 females and 3 males all showed exceptionally good growth. They were all fertile and the females had repeated litters but very little success in lactation. Only 2.6 per cent of the young born were weaned. Two of the animals showed badly diseased lungs at autopsy and 2 had inflamed intestines.

Of the 3 second generation females, only one was fertile and this animal had some success in lactation. One of the 2 second generation males was sterile, which accounts for the failure of the other 2 females to reproduce.

Beef Kidney, Ration 145. Growth was not quite so good as on the liver ration. The 8 females were all fertile but nearly all had their first litters late and 3 were infertile late in life. Only 4.8 per cent of the young born survived. The two second generation females and one male were all fertile.

DISCUSSION OF RATIONS IN SERIES V

It would appear that dried kidney contains a little less vitamin B than dried liver. The fact that all of the animals made normal growth shows, however, that liver and kidney both contain sufficient vitamin B for normal growth when fed at a 20 per cent level.

Since the requirements for vitamin B are so much greater for lactation than for growth, the effect of removing the yeast from rations 134 and 144 is evident in the animals on series V rations.

Rations 134, 135, 144 and 145 are compared in Table II. In the Series V rations there is an increase in the number of young born but a distinct decrease in the per cent of young which were weaned.

TABLE II
RESULTS WITH AND WITHOUT YEAST

Ration 20% prot. no lard, c.l.o. daily after 135 days	No. females	Av. age at birth 1st lit. days	Av. No. lit. per female to 255 days	Av. No. born per female	Per cent that lived	Remarks
134, 50% yeast	9	117	2.9	19.1	12.2	2nd generation 50% fertile
135, No yeast	8	92	4.3	28.5	1.3	2nd generation partly fertile
144, 50% yeast	8	149	2.3	14.5	7.7	2nd generation partly fertile
145, No yeast	8	185	2.5	19.0	4.6	2nd generation fertile

Series VI—High Protein, Test for Vitamin A

The rations of series VI were planned for two reasons. First, it seemed desirable to make a comparison of the protein foods for vitamin A content. Second, since the addition of the cod liver oil daily to the rations of Series IV and V had such a beneficial effect on reproduction, it seemed desirable to omit it entirely, relying on the protein foods themselves for vitamin A and radiating[4] the animals to provide vitamin D. Since beef muscle is known to be low in vitamin A that meat was omitted from the series. The rations were as follows:

Composition of Rations in Series VI
(20% protein, no lard, no c.l.o., 5% ash)

Ration No.	Product (powdered)		Starch	Salts	Yeast
136	Liver	32.2	58.8	4.0	5.0
146	Kidney	28.0	63.0	4.0	5.0

The animals in this series showed unusual success in reproduction but not in lactation. In the attempt to determine the missing factor or factors, an addition of 2 per cent of wheat germ oil was first made to the rations at the time the animals were about 175 days of age. A little later 3 per cent more of yeast was added, making a total of 8 per cent. The results of these modifications will be taken up in the discussion of each ration.

Beef Liver, Ration 136. The 9 females and 3 males on this ration nearly all showed growth which was above normal. Three of the females had no litters before the oil addition and one was sterile throughout life. Only one litter of two was raised before oil. After oil and yeast the same female raised a litter of seven. Oil alone cannot be said to have improved lactation. After the yeast addition, 3 females together raised 11 young. Two of the males were fertile but one was questionable. At autopsy 2 of the animals were shown to have slight fatty degeneration of the liver, similar to that found in the first lot on ration 130. Three had badly diseased lungs.

The two females and one male from the second generation showed very good growth and all were fertile. One female raised a litter of 4 following oil and yeast.

Beef Kidney, Ration 146. The 8 females and 3 males on this ration all showed growth above normal. All but 2 of the females were fertile before the oil addition. One of the 2 which were sterile before oil, had lung disease and was anesthetized. The other had a litter following the oil. The remaining 6 had repeated litters of large size but only 2 raised young. There was no improvement after oil alone, but after oil plus yeast, 2 females together raised 15 young. Results might have been better had there not been so much lung disease.

Discussion of Rations in Series VI

Interpretation of results on the rations of this series is complicated by the fact that infections were so prevalent among the animals. This susceptibility to disease has been noted by a number of workers to exist among animals deprived of adequate amounts of vitamin A (Steenbock,

[4] The animals were radiated twice a week for 3 minutes at a distance of three feet. The lamp used was a Hanovia Quartz Mercury Arc.

1923; Sherman, 1925). One fact is very evident, however, that by omitting cod liver oil entirely, ration 146 was made better for reproduction than ration 144, which contained cod liver oil. The improvement was noted especially in the average number born per female and in the average number of litters per female up to the age of 140 days, as shown in Table III.

TABLE III

RESULTS WITH AND WITHOUT COD LIVER OIL

Ration	No. females	Av. age at birth 1st lit. days	Av. No. lit. per female to 140 days	Av. size litter	Av. No. born per female	Per cent that lived
134, Liver, +c.l.o.	9	117	1.20	6.4	7.7	0.0
136, Liver, no c.l.o.	9	114	.78	8.0	6.2	3.6
144, Kidney, +c.l.o.	8	149	.87	6.9	6.0	0.0
146, Kidney, no c.l.o.	8	106	1.40	9.1	12.5	8.0

The animals on ration 136 were no more fertile than those on 134. The comparison can be carried only up to the time the animals were 140 days of age, on account of the fact that wheat germ oil was added to the rations of Series VI when the animals were about that age.

It was rather surprising that reproduction was better on ration 146 than on 136 and there seems to be no explanation for the fact except that kidney protein is superior to liver protein.

Lactation was practically a failure on the rations of this series. The reason for this is a matter for speculation. The animals seemed to have enough vitamin A for growth and reproduction but it may be that an additional supply is needed for lactation, as is true of vitamin B, especially the heat-labile fraction. The fact that so many of the females had diseased lungs may also account for the poor lactation. Table III really gives a wrong impression as to the success in lactation on rations 134 and 144, since the figures were compiled before the females had borne very many litters. As will be seen from Table III, there was some success in lactation on these rations. However, that function was not nearly so good as it should have been, and it is very probable that those rations would have been improved for lactation by the addition of more yeast, just as the Series VI rations were.

If freedom from infection on a ration is an indication of the presence of adequate amounts of vitamin A, it can be said that ration 136 (liver) appears to have contained a little more than the other rations in the series. This agrees with the work of Sherman and Boynton (1925). Those

workers state that rat liver contains 200 to 400 times as much vitamin A as rat muscle.

Series VII—Raw Protein, No Added Lard

Finally, in order to determine whether cooking has any detrimental effect on the nutritive value of the meats, it was thought desirable to try a series of rations containing them in the raw condition. In planning the rations it was decided that as much of the raw meats should be used as would be necessary to provide 15 per cent of protein if they were in the dry form. The amounts of the other constituents were then calculated by assuming that the meat was dried. The rations were as follows:

Composition of Rations in Series VII
(15% uncooked protein, no lard, 5% ash)

Ration No.	Raw protein		Starch	Salts	Yeast	C.l.o*
	Food	gms.	gms.	gms.	gms.	gms.
127	Round	71.4	68.2	4.5	5.0	2.0
137	Liver	68.2	64.8	4.0	5.0	2.0
147	Kidney	87.2	67.6	4.0	5.0	2.0

* Daily

In feeding these rations, the raw meats plus the cod liver oil were added daily to the dry basal mixture, which was made up in large quantities. The meats were first carefully freed from visible fat and ground finely in a meat grinder.

Beef Round, Ration 127. All but one of the 8 females and all of the 3 males on this ration showed normal growth up to 100 days of age, but after that time several showed a decline. Reproduction records were poor. Four of the females were infertile, one had 2 litters, and the other 3 had one litter each. These results in the females may have been partly due to the males, 2 of which were apprently infertile, although their testes were 85 and 88 per cent of the normal weight. Many of the animals showed lung disease.

Beef Liver, Ration 137. Growth on this ration was normal, or above, with one exception. Two of the 8 females were infertile. Both of these showed badly diseased lungs and one also had a diseased liver and uterus. In fact, all but one of the animals on this ration developed some lung disease. Two of the 3 males proved to be infertile, which partially accounts for the poor fertility of the females. The growth of the one fertile male was unusually good and this animal was free from infections. Lactation was fair, 30 per cent of the 56 young surviving.

Two females and one male from the second generation were kept on the ration. Growth was above normal but the animals had to be discarded before the reproductive period had begun.

Beef Kidney, Ration 147. The 9 females and 3 males on this ration all showed excellent growth. The females were all fertile but the records of three were poor, due to the fact that they were mated with the one male which proved to be infertile. A total of 110 young were born, of which 33 per cent were weaned.

Five females and 3 males from the second generation were kept on the ration. Two of the females had litters and 2 of the males were shown to be fertile. The others were anesthetized while still too young for reproduction.

Discussion of Rations in Series VII

The poor results on raw beef muscle were probably partly due to the large amount of tough connective tissue, which caused poor assimilation of the meat. At least this explanation is suggested by the work of Mitchell (1927), who found that the feeding of connective tissue with the muscle tissue of pork tenderloin caused a depression in the biological value of the enderloin. It was expected that this ration would show considerably better reproduction than ration 120, which also contained 15 per cent protein with added lard and cod liver oil mixed into the ration. Such was not the case, however.

Reproduction on ration 147 (kidney) was considerably better than on 137 (liver), and here again the most probable explanation is that kidney protein is superior to liver protein. Moreover, this superiority possibly appears to better advantage in the raw meats than in the cooked and dried preparations.

A comparison of the liver and kidney rations of Series I and VII, up to the time the animals were 150 days of age, gives results as follows:

	Ration	Av. No. born per Female	Per cent which lived
Liver	130 (1st lot)	6.0	50.0
	130 (2nd lot)	9.3	10.7
	137	4.1	30.3
Kidney	140	2.3	0.0
	147	5.8	19.2

It is difficult to explain why reproduction on ration 137 was not so good as on ration 130. It can be said, however, that considering the first and second generation animals throughout life, ration 137 was better because fertility lasted longer. The second generation male on ration 137 had normal testes weight, while the second generation males on ration 130 all showed degeneration.

Ration 147 was considerably better than 140, both for reproduction and lactation. The difference in lactation is no doubt partly due to the fact that in ration 140 there had been some destruction of vitamin B in the preparation of the dried kidney. The fact that the second generation animals on ration 147 were fertile is another point in favor of this ration. This was probably the result of omitting the lard and cod liver oil from the main bulk of the food.

Summary and Conclusions

In this comparative study, beef round, liver and kidney, in both cooked and raw forms, were fed to rats in order to determine the relative efficiency

of these foods as sources of protein and accessories, primarily for reproduction and lactation. The rations contained sufficient amounts of the meats being tested to supply 15 and 20 per cent protein. Various supplements were used, depending on the particular food constituent being tested for.

Variations in the proportions of protein and fat in the ration involved changes in the amount of lard used as a source of added fat. Rations in which lard was omitted entirely, proved better for reproduction than those which contained even a small amount. Cod liver oil was also found to have a detrimental effect on reproduction when mixed into large quantities of the rations. Therefore, in all of the later rations this oil was either added daily to small amounts of the rations or omitted entirely and partly replaced by radiation.

When no supplements of vitamin E were made, the meats themselves being relied upon as a source of this vitamin, the rations, containing only 15 per cent of protein with lard and cod liver oil, thus contained two variable factors since these added fats had a destructive effect on the vitamin. This made the interpretation of results as to the relative value of the proteins, as such, rather difficult. When, however, vitamin E was supplied in adequate amounts, the superiority of kidney protein seemed to be clearly indicated. Liver protein ranked next, and muscle last.

When the difference in the quality of the proteins was largely ruled out by feeding them at a 20 per cent level, the vitamin E content of the dried meats was found to decrease in the following order: Round, liver, kidney.

It seems probable that 5 per cent of yeast does not provide enough vitamin B (or B_1) for highly successful lactation in those rations containing dried meats. This was indicated by the fact that lactation on ration 120 (muscle) was distinctly improved by addition of the alcoholic extract of ether-extracted wheat embryo, which is rich in vitamin B (or B_1). Additional yeast, together with wheat germ oil, also caused improvement in lactation on rations 136 (liver) and 146 (kidney). The superiority of liver over the other meats for this function was no doubt due to its content of vitamin B, particularly the heat-labile fraction.

It is thus difficult to say which of the three meats is best for reproduction and lactation, unless it is specified in what form and amount the food is to be fed, and with what supplements. If fed in the dried form at a 20 per cent protein level in a ration containing no substances which would

have a destructive effect on vitamin E, beef round probably requires supplements of minerals and vitamins A, B, and D. Beef liver and kidney probably require in addition a supplement of vitamin E. In the raw state beef round is not easily assimilated by rats, but liver and kidney appear to have a higher food value when raw than when cooked.

It is not known whether cooking has any detrimental effect on the proteins of meat, but a large part of the vitamin B (antineuritic) must be destroyed. Of the raw meats, kidney would seem to require the fewest supplements for successful reproduction and lactation.

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THE EFFECT OF VARYING AMOUNTS OF MENHADEN OIL IN THE DIET ON THE COMPOSITION OF THE BODY FAT OF THE WHITE RAT. THE STORAGE OF HIGHLY UNSATURATED FATTY ACIDS

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THE influence of the fat of the diet on the composition of mammalian body fat is now generally recognized. Excellent reviews of this subject are given by Leathes (1) and by Lewkowitsch (2). While in general the fat from animals of the same species is fairly typical, this is due more to the fact that individuals of the same species live under similar conditions of diet and environment. Changes in these, particularly in the diet, may profoundly alter the composition of the body fat. The earlier work on this subject will not be reviewed here. However, several investigations pertaining thereto have appeared recently. Ellis and Isbell (3), in a study of the soft pork problem, produced lards with iodine numbers of 52 to 97 by the use of controlled experimental diets. The feeding of soy bean and peanut oils produced especially unsaturated fats. Anderson and Mendel (4), in a detailed investigation of the effect of diet on the body fat of white rats, obtained specimens of fat with iodine numbers from 55 to 93. They found, in agreement with earlier investigators, that previous depletion of the store of body fat by starvation resulted in greater changes. Eckstein (5) studied the effect of five diets on the composition of the lipids of the white rat and obtained lipids with iodine numbers from 47 to 88. Myristic and oleic acids from the diet were deposited while butyric acid was not.

In a previous report from this laboratory (6) it was shown that when a diet containing 20 per cent whale oil was fed to the white rat, equilibrium was established between the diet and the depot fat in from four to six weeks. The presence of increased amounts of highly unsaturated fatty acids in the stored fat was demonstrated by means of their ether-insoluble bromine addition products. Unfortunately, the whale oil used gave bromides which contained very close to the theoretical amount of bromine

for octobromoarachidic acid (67.78 per cent) so that it was uncertain whether the acid deposited was arachidonic acid, or a mixture of the whale oil acids. Usually the fish oil fatty acids yield bromides which contain about 2 per cent more bromine (69–70 per cent) than octobromoarachidic acid. Hence they may be distinguished readily from arachidonic acid. Since they apparently are deposited without difficulty in mammalian fat and are detected easily, fish oils may be used in studying certain phases of fat metabolism.

The amount of arachidonic acid present normally in the body fat of the white rat is very small (5), not over a fraction of a per cent. In several instances in this laboratory, specimens of normal rat fatty acids have yielded no ether-insoluble bromides, thus showing the absence of this acid.

In the present work, the effect of varying amounts of menhaden oil in the diet of white rats on the composition of their fat has been followed. Menhaden oil contains acids of the C_{16}, C_{18}, C_{20} and C_{22} series with from three to six double bonds (7). The oil used in this experiment was purchased on the market as refined menhaden oil and had an iodine number of 169.7. Its fatty acids gave 54.8 per cent of their weight of ether-insoluble bromides, containing 69.14 per cent bromine. When the rats were fed diets containing 0 to 30 per cent of this oil, increasing amounts of highly unsaturated acids were deposited in the body fat, the results showing 1.3 per cent in the controls on a diet containing 10 per cent olive oil, to 17.2 per cent in the rats on 30 per cent menhaden oil diet. In every instance the bromides isolated from the depot fatty acids contained more bromine than those from the acids of the original oil, the excess being sufficient to suggest either that the more unsaturated fatty acids of higher molecular weight were selectively deposited or that desaturation took place in the body.

EXPERIMENTAL PART

Description of Experiment. Six groups of seven adult rats each were partially starved over a period of four weeks by feeding insufficient amounts of the following control mixture,—

Whole wheat flour	653 gms.
Casein	160 "
Milk powder	160 "
NaCl	11 "
Chalk, precipitated	16 "

To each kilogram of dry mixture was added 4 cc. of cod liver oil and sufficient water to make a workable dough. The dough was spread in thin

layers and partially dried in air. The small amount of cod liver oil, added for its vitamin content to the basal mixture, was believed to be insignificant so far as its effect on the body fat was concerned. In the light of the results shown by these experiments, however, this oil may account for part at least of the 1.3 per cent unsaturated acids found in the depot fat of the control animals. At the end of the period of partial starvation, the six groups were placed on the diets described in Table I. The diets

TABLE I

Diet Per cent	Wt. dry mixture	Wt. Oil	Wt. Water	Per cent total calories as fat
	grams	grams	grams	
control	1000	111 olive oil	400	22
5	1000	52.6 menhaden oil	450	12
10	1000	111 " "	400	22
15	1000	176.4 " "	400	31
20	1000	250 " "	350	39
30	1000	428.6 " "	300	52

were kept cold and protected from oxidation as much as possible. To be more certain that oxidation did not occur, during the latter part of the experiment each diet was prepared every other day in sufficient quantities to be consumed relatively fresh. A record was kept, so far as possible, of the amount eaten by each group of rats during six weeks of feeding, these data being given in Table II.

TABLE II

FOOD AND OIL CONSUMPTION

		5%	10%	15%	20%	30%
Total weight eaten, gms.	3942	3692	4098	3970	3093	2458
Weight eaten on dry basis, gms.	2838	2547	2950	2858	2381	1969
Weight of menhaden oil consumed	283	127	295	428	476	590
	(Control olive oil)					

The weight changes during the course of the experiment are given in Table III.

The physical condition of all of the rats at the end of the experiment was good. No evidence of any deleterious effects was observed even in the 30 per cent diet, in which over half of the total calories ingested were derived from menhaden oil. During the period no general increase in weight was observed.

TABLE III
AVERAGE CHANGES IN WEIGHT DURING EXPERIMENT

Average weight	Control	5%	10%	15%	20%	30%
			grams			
At beginning	224	220	228	237	232	229
After four weeks partial starvation	197	176	186	198	205	201
After one week on diet	230	203	221	221	230	207
After six weeks on diet	231	209	238	229	237	225

RESULTS OF FEEDING MENHADEN OIL

At the end of the period of experimental feeding, five rats from each group were killed with chloroform, skinned, and the body fat, including that in the abdominal cavity, was removed and rendered by heating in a small flask at 130° for an hour under reduced pressure. The fat was taken up in ether, and the residue ground up and extracted with ether. The ether was removed by warming under reduced pressure. The product was kept in a small bottle under carbon dioxide. The oils were pale amber colored. On standing at room temperature, stearin settled out, the largest amount forming in the 5 per cent diet.

The oils were analyzed by the usual methods. For iodine number the Hanus method with one-half hour reaction was employed. The polybromide number was determined by brominating a 2 to 3 gram sample of oil or fatty acids in a weighed 50 cc. centrifuge tube in cold dry ether. An excess of bromine was added slowly, and the reaction allowed to stand at least four hours. The ether was removed by centrifugation, more ether was added and the bromides thoroughly stirred, and the tube again centrifugated. This washing was repeated four times, and the final product, consisting of pure white bromides, was dried at 50° and weighed. Bromine was estimated by the Parr peroxide bomb method. The per cent of highly unsaturated fatty acids found in the fatty acids was calculated by a method described previously for arachidonic acid (8). The polybromide number of the pure highly unsaturated acids of menhaden oil has been found to be 105 (7). A polybromide number of 10.19 would therefore represent $10.19 \times 100/105$ or 9.7 per cent of highly unsaturated fatty acids. This method is admitted to be only approximate but the writer believes it to be more accurate than merely to calculate the acid content of the bromides, which gives a result far too low.

Results of the analyses of the oils and the corresponding fatty acids obtained from them are given in Table IV.

TABLE IV
CHARACTERISTICS OF BODY OILS

Diet Per cent of fat	Fats				Fatty Acids			
	Sapon. number	Iodine number	Poly-brom. number	Refrac. index	Iodine number	Poly-brom. number	% Br in bromides	% HUFA*
Control	197.3	73.16	.56	1.4648	82.59	1.42		1.3
5	199.3	82.71	5.89	1.4663	90.39	10.19	70.09	9.7
10	197.3	94.67	14.95	1.4691	98.05	15.16	69.58	14.4
15	194.4	99.01	15.73	1.4697	99.60	15.63	69.87	14.9
20	196.0	97.24	18.81	1.4671	104.7	16.94	69.65	16.1
30	196.0	103.35	18.80	1.4710	104.0	18.08	70.02	17.2
Menhaden oil	191.9	169.7		1.4828	175.9	54.8	69.14	52.2

* Highly Unsaturated Fatty Acids calculated from polybromide number.

DISCUSSION OF RESULTS

Examination of the analytical results in Table IV shows conclusively that addition of menhaden oil to the diet of the white rat results in deposition of the characteristic highly unsaturated fish oil fatty acids in quantities roughly proportional to the amount of oil in the diet. The changes in saponification number were not so great as might be expected, since this constant for menhaden oil is appreciably less than it is for rat fat. However, the iodine number of the fat varied from 73 in the control with 10 per cent olive oil in the diet to 103 with 30 per cent menhaden oil. The iodine number of the fatty acids rose from 82 to 104 under the same conditions. The polybromide number of the control fatty acids was 1.42. If calculated as arachidonic acid, this would represent 1.8 per cent of this acid. (The polybromide number for pure arachidonic acid is 80.) If calculated as the highly unsaturated acids of menhaden oil, the content of these would be 1.3 per cent. The polybromide number rose rapidly with increase of fish oil in the diet to 18.08, representing 17.2 per cent of highly unsaturated acids.

The data in Table V compare the per cent highly unsaturated acids in the body fat with that of the diet (calculated). It is interesting to note that with the 5 per cent diet the ratio of the former to the latter is about 4:1. This ratio decreased rapidly to about 1:1 with the 30 per cent diet. In other words, there appeared to be a concentration of unsaturated acids in the body fat which was more pronounced in the lower diets. The amount

TABLE V

A COMPARISON OF THE CONTENT OF HIGHLY UNSATURATED FATTY ACIDS OF THE BODY FAT AND OF THE DIET

Diet Per cent of Oil	Per cent highly unsaturated fatty acids		
	In Body Fat	In Diet	Ratio
Control	1.3	0.0	—
5	9.7	2.6	4:1
10	14.4	5.2	3:1
15	14.9	7.8	2:1
20	16.1	10.4	1.5:1
30	17.2	15.7	1:1

of highly unsaturated acids deposited became nearly constant in the 20 and 30 per cent diets.

From a theoretical standpoint, the results of analysis of the bromides from the body fatty acids are particularly interesting. In every instance they contained from 0.50 to 1.0 per cent more bromine than the bromides obtained from the original menhaden oil fatty acids. The deposited highly unsaturated acids were therefore more unsaturated than those in the fish oil. The theoretical iodine number of the acids in a bromide containing 69 per cent bromine is 352, and for a bromide containing 70 per cent bromine, 370. It is known that the highly unsaturated acids of menhaden oil contain acids from the C_{16} series with three double bonds (hexadecatrienoic) to C_{22} with six double bonds (docosahexenoic) (7). When these acids are fractionated, the bromine content of the bromides of the fractions increases with boiling point. Two explanations may be offered, therefore, to account for the increase in unsaturation. The rat may metabolize the C_{16} and C_{18} acids of the fish oil more readily than the C_{20} and the C_{22} acids, thus removing those of lower molecular weight and of lesser unsaturation and depositing the higher molecular weight, more unsaturated acids. The second possibility is suggested by the Leathes desaturation theory. The highly unsaturated acids may be still further desaturated in the body with the introduction of more double bonds. The resultant acids would give the constants found.

With such small quantities of material, and with only the bromine analyses with which to work, it is of course impossible to settle the question as to the nature of the changes taking place in the acids. It is our hope to get further light on the question by feeding larger animals, such as the hog, where large enough samples of depot fat may be obtained to make it possible actually to isolate the deposited highly unsaturated acid for comparison with those obtained from the fish oil that is fed.

SUMMARY

1 The characteristic highly unsaturated fatty acids of menhaden oil are deposited in the body fat of the white rat when these acids are included in the diet.

2 Diets containing 5 to 30 per cent of menhaden oil resulted in a deposition of highly unsaturated acids from 9.7 to 17.2 per cent.

3 In the diets studied, the content of highly unsaturated acids of the depot fat was always higher than that of the diet.

4 The highly unsaturated acids deposited were more unsaturated than those from the original menhaden oil.

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Editorial Review[1]

THE CHEMISTRY OF VITAMIN D[2]

IN THE attempts which have been made to identify the vitamins, there has been found in irradiated ergosterol, a substance or mixture of substances, which can be more nearly identified with vitamin D than can any other substance with any other vitamin. Vitamin D is concerned specifically with the utilization of calcium and phosphorus in the body and its absence from the diet leads frequently to the condition known as rickets.

It was first shown experimentally in 1922 by McCollum and coworkers (1) that such a vitamin exists. By aerating cod liver oil, these investigators found that vitamin A which was then known to be present in the oil, was destroyed while another substance was left which played "an important rôle in bone growth". Soon after this, it was found that the non-saponifiable portion of the cod liver oil contained the vitamin (2).

It had been shown by Huldshinsky (3) in 1919 that the ultra-violet rays of the sun were effective in the treatment of rickets and in 1924 Hess (4) and Steenbock (5) almost simultaneously demonstrated that the feeding of a food which had been exposed to ultra-violet light was as effective as irradiating the animal itself. These investigators showed that most natural foods could be endowed with antirachitic value by irradiation with ultra-violet light. If vegetable oils were irradiated and made active antirachitically, their unsaponifiable fractions possessed the antirachitic properties just as in the case of cod liver oil. Old oils could not be activated by irradiation with ultra-violet light nor was it possible to activate their unsaponifiable fractions.

Since phytosterol is the main constituent of the non-saponifiable portion of vegetable oils, and cholesterol an important part of the non-saponifiable portion of cod liver oil, these two substances were next studied in many laboratories. When carefully purified by repeated crystallization, both were made active antirachitically on irradiation with ultra-violet rays shorter than 315 mμ (6, 7).

Hess and coworkers (8) next showed that the cholesterol which had been irradiated could be separated into an active and an inactive fraction, by

[1] As stated in the first issue of this journal, the term Editorial Review signifies not that the writer necessarily is an editor, but that the review has been read and criticized by at least four members of the Editorial Board.

[2] The author, Dr. Florence L. MacLeod, is Research Assistant, Department of Chemistry, Columbia University.

precipitation with digitonin, the activity being present in the non-precipitable fraction. Likewise from cod liver oil, on precipitating the cholesterol from the non-saponifiable fraction, a more active fraction was obtained. Koch and coworkers (9) and Hess and coworkers (8) both found that if cholesterol or the non-saponifiable fraction of cod liver oil were extracted with liquid ammonia, the soluble fraction had increased activity.

The different investigators now began to feel that the activity of the cholesterol was probably due to an impurity rather than to cholesterol itself. Windaus and Hess (10) pointed out that a very small part of the cholesterol underwent the change for the melting-point and the composition of the irradiated product were unchanged. They stated that the only difference in the product besides its antirachitic potency was a decrease in the absorption of ultra-violet light between 280 and 300 mμ on irradiation. While they were working Rosenheim wrote to Windaus saying that he was convinced that cholesterol contained a small amount of impurity. Pohl and Heilbron, Kamm and Morton came to the same conclusion at approximately the same time.

Rosenheim and Webster (11) showed that after irradiation they could recover at least 99.9 per cent cholesterol by precipitation with digitonin. The active substance was left unprecipitated. They also showed that an isomer of cholesterol, allocholesterol, could not be activated and further that if cholesterol were converted into the dibromide and reduced again to cholesterol, the cholesterol obtained could not be activated. So they concluded that the precursor of vitamin D was not cholesterol but a substance associated with it and one which followed cholesterol in all its stages of purification by the usual methods (esterification, saponification and recrystallization).

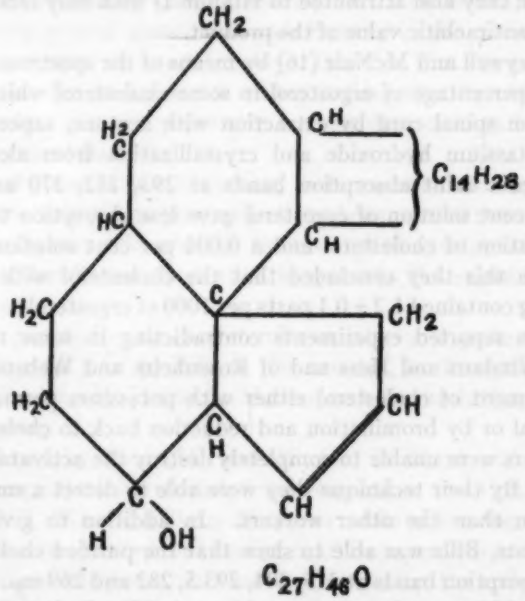
Heilbron, Kamm and Morton (12) were able by recrystallization of cholesterol from ethyl acetate to concentrate the active substance in the least soluble fraction and to show that this fraction had more clearly defined absorption bands than the original cholesterol. They felt that this was strong evidence that the substance showing the absorption bands in the ultra-violet region of the spectrum was different from cholesterol and was probably the precursor of the vitamin. Windaus and Hess (10) were able to show similarly that cholesterol purified by way of the dibromide did not exhibit antirachitic activity on irradiation with ultra-violet rays and did not exhibit the characteristic absorption bands. They also freed the cholesterol from the "provitamin", as they called the impurity, by prolonged boiling with blood charcoal, and by treatment with potassium

permanganate, and were able to concentrate it, in the least soluble fraction, by recrystallization from acetic acid, and by high vacuum distillation.

They found that the "provitamin" was precipitated by digitonin before irradiation and that it must be an unsaturated compound for if it were hydrogenated, it no longer could be activated by ultra-violet light.

The sensitiveness of the "provitamin" to oxidative processes is characteristic of one of the known sterols, ergosterol. Tanret (13) who described ergosterol in 1908 had studied its behavior under the influence of light and air as evidenced by change in color, fall of melting point and change in optical activity. The behavior of the "provitamin" suggested to Windaus that it possibly was ergosterol, which is similar in structure to cholesterol but has three double bonds instead of one and like cholesterol has one OH group.

The latest formula which Windaus (10) gives for cholesterol is:



$C_{27}H_{46}O$

All that is known of ergosterol is that there are two more double bonds in its molecule but it is not known where they are. Its empirical formula then is $C_{27}H_{42}O$. Tanret originally prepared it from the fungus, ergot, but at present it is commonly obtained from yeast.

Ergosterol, one part in 600,000 parts of absolute alcohol, was found by

Windaus and Hess to give the same absorption spectrum as a one per cent solution of cholesterol. A pure cholesterol, free from the "provitamin", was mixed with 1/60 per cent of ergosterol and a product was obtained which was not separable from cholesterol by crystallization, melting-point, optical activity or composition. The irradiated ergosterol was much more potent antirachitically than cholesterol.

Pohl (14), working with the same materials as Windaus, showed also that the absorption spectra of cholesterol and ergosterol were similar. Pohl found that after irradiation for 25 minutes the ergosterol showed an absorption band only at about 240 mμ, instead of exhibiting maxima at 293, 280 and 270 mμ, and attributed this band to vitamin D. Morton, Heilbron and Kamm (15) studied at half-hour intervals the changes in the absorption spectrum brought about by the irradiation of ergosterol in alcohol solution. After two and a half hours they obtained an absorption band at 247 mμ which they also attributed to vitamin D with only incidental tests to prove the antirachitic value of the product.

Bills, Honeywell and McNair (16) by means of the spectroscope showed roughly the percentage of ergosterol in some cholesterol which they had obtained from spinal cord by extraction with acetone, saponification in alcoholic potassium hydroxide and crystallization from alcohol. The cholesterol gave faint absorption bands at 293, 282, 270 and 260 mμ. A 0.003 per cent solution of ergosterol gave less absorption than a three per cent solution of cholesterol and a 0.004 per cent solution somewhat more. From this they concluded that the cholesterol with which they were working contained 1.2 ± 0.1 parts per 1000 of ergosterol.

They then reported experiments contradicting in some respects the reports of Windaus and Hess and of Rosenheim and Webster. By prolonged treatment of cholesterol either with potassium permanganate or with charcoal or by bromination and reduction back to cholesterol, Bills and coworkers were unable to completely destroy the activatability of the cholesterol. By their technique they were able to detect a smaller degree of activation than the other workers. In addition to giving positive biological tests, Bills was able to show that the purified cholesterol gave five faint absorption bands at 315, 304, 293.5, 282 and 269 mμ. From these results he and his coworkers were unable to decide whether the activation of the cholesterol was entirely due to ergosterol. The absorption bands at 293.5, 282 and 269 mμ would indicate that it was,whereas they felt that it was difficult to believe that bromination which destroyed more than 99 per cent of ergosterol when applied once, should fail to destroy the remainder when applied a second time. Jendrassik and Keményffi (17)

reported at about the same time that cholesterol could still be activated after purification by way of the dibromide and they believed that ergosterol was not the only substance capable of being activated.

Koch, Koch and Lemon (18) have recently published some experiments confirming these results of Bills. They found that on purifying cholesterol by the bromine treatment or by a triple treatment with potassium permanganate, the two products upon irradiation induced calcification in dosages 70 to 30 times as large as that required of the original cholesterol. The two products showed no absorption bands corresponding to ergosterol and showed no general absorption in the ultra-violet region.

These investigators do not believe this low activatability to be due to a trace of ergosterol. When their purified products were heated slightly above the melting point under conditions that avoided appreciable oxidation, every one of the purified products was practically 25 to 70 times as active as the presumably pure cholesterol. With the increase in activatability a strong general absorption was observed in the ultra-violet region but since there were no bands whatever they feel that the potency of the product is not due to ergosterol. They believe that "provitamin D activity" may be a general property in varying degrees of various sterols or of certain forms of those sterols.

In spite of these rather disturbing findings, most of the work which has been done recently on this subject has been carried out on ergosterol, to see what changes take place in the absorption spectra under different conditions of irradiation, and what happens to the ergosterol molecule on irradiation.

Spectroscopic studies on cholesterol and later on ergosterol have been made for the most part by Bills in this country, by Rosenheim, Webster and Bourdillon and by Heilbron, Morton and Kamm in England and by Pohl in Germany.

It was shown by Pohl (14) and Morton, Heilbron and Kamm (15) that when ergosterol is irradiated for some time the three absorption bands at 293.5, 282 and 270 mμ disappear while a new band appears at 247 mμ. As was pointed out earlier in this paper, these investigators assumed without adequate biochemical control that the substance giving the absorption band at 247 mμ was vitamin D.

Bills and coworkers (19) undertook similar experiments in which they studied the effect of irradiation with ultra-violet light on the absorption spectrum of ergosterol over a long period of time, conducting parallel spectroscopic and feeding tests. The ergosterol was of exceptional purity giving an $[\alpha]_D^{200} = -132°$ in chloroform which would indicate a high

degree of purity, one gram being dissolved in a liter of optically pure 95 per cent alcohol. The solution was activated in a homogeneous quartz absorption cell, 2 cm. deep, completely filled. A water-cooled mercury lamp was used and the light was reasonably constant with uniform voltage and temperature. The absorption curves were made with a Hilger quartz spectrograph. Fresh cellfuls were used for irradiating the solutions for each period of exposure. A portion of each irradiated alcoholic ergosterol solution was used for photographing the absorption spectra while the rest was used for the biological tests.

The results of this investigation were as follows:—

Non-irradiated stock solution. This solution gave absorption bands with maxima at 270 and 282 mμ. The band at 293.5 mμ was evident, a fourth band at 260 mμ which had been detected by Bills and MacNair (16) in previous experiments was too faint to be recognized. The non-irradiated solution had no antirachitic acitivity.

Irradiated 7-1/2 minutes. The absorption curve resembled that of the non-irradiated solution with the maxima a little lower. There was no evidence of any new absorption band yet the tests revealed the development of antirachitic activity 150,000 times as great as that of cod liver oil.

Irradiated 15 minutes. There was a slight change in the absorption curve but no evidence of a new band. The activation nearly reached its maximum at this point, the potency being 225,000 times that of cod liver oil.

Irradiated 22-1/2 minutes. The absorption band at 270 mμ broadened but there was little change in the absorption curve. With this length of time of irradiation the activation reached its highest point with a potency 250,000 times that of cod liver oil.

Irradiated 30 minutes. The maximum at 293.5 mμ disappeared, that at 282 mμ was still conspicuous and that at 270 mμ showed a distortion which indicated the development of a new region of absorption in the shorter wave-lengths. The potency declined to 200,000 times that of cod liver oil.

Irradiated 1 hour. The maximum at 282 mμ nearly disappeared, that at 270 mμ was barely evident and the new maximum began to become distinct at about 252 mμ. The potency continued to decline.

Irradiated 2 hours. All the absorption maxima of ergosterol disappeared. The new band became more intense with its maximum at about 250 mμ. The antirachitic potency declined to 50,000 times that of cod liver oil.

Irradiated 3 hours. Coincident with the development of the new band at 248 mμ in its greatest intensity, the antirachitic potency almost disappeared.

Irradiated 4, 5, 7-1/2, 10 and 15 hours. Between 3 and 4 hours, the absorption band at 248 mμ faded slightly; during 4 to 7-1/2 hours no further change was observed; after 7-1/2 hours the fading resumed. The antirachitic potency never reappeared.

From this it is evident that the photochemical reaction product which exhibits the absorption band at 248 mμ is not vitamin D. The appearance of this band coincides not with the development but rather with the destruction of antirachitic potency. Bills says that it is a by-product of the activation of ergosterol or a degradation product of the vitamin.

The activation curve considered with the absorption curve shows that the antirachitic potency is developed rapidly reaching a maximum when

about 73 per cent of ergosterol is still present. The decline in potency commences at the point where on account of a depleted reserve of ergosterol and consequent decline in the rate of formation of the vitamin, the decomposition overtakes the formation.

When one cellful of solution was used and samples were withdrawn from time to time, leaving more and more air in contact with the residual liquid, the band at 248 mμ developed and faded much more rapidly. The amount of oxygen in the air contained in the partly filled cell was sufficient for considerable oxidation. The oxidation causes a destruction in the 248 mμ band and does not affect the formation of vitamin D.

The substance showing the absorption band at 248 mμ is clearly related to ergosterol and vitamin D. Bills points out that isoergosterol, described by Reindel, Walter and Rauch (20) exhibits just such a band. The isoergosterol was obtained by the addition and then the removal of hydrochloric acid from the ergosterol molecule. The absorption of isoergosterol is from 220 mμ to 270 mμ with a maximum at 248 mμ. This band fades also on prolonged irradiation. It is not at all certain that isoergosterol and the substance formed by the irradiation of ergosterol are identical but Bills feels that with the formation of vitamin D by light a substance is formed which must have a molecular configuration not unlike isoergosterol. This same suggestion, that the absorption band at 248 mμ is due to isoergosterol, has been made by two other investigators, van Wijk and Reerink (25).

Of interest in connection with this paper by Bills is a report of Rosenheim and Webster (21) in which they showed, by precipitation with digitonin, that after a half hour of irradiation, 90 per cent of unchanged ergosterol is left whereas after 4 hours there is 10 per cent of ergosterol and the antirachitic potency is the same. The conditions of their experiments were probably different and not so carefully controlled as those of Bills but the results do indicate that the activity of the reaction products do not continue to increase with the disappearance of ergosterol.

Bourdillon, Webster and coworkers (22) hold the theory that the ultra-violet irradiation of ergosterol produces three substances in succession. The first, which they call A, shows intense absorption for wavelengths of 250 to 310 mμ with a maximum absorption at 280 mμ. The second, called B, shows intense absorption at 240 mμ and no antirachitic power. The final product shows little or no absorption or antirachitic power. They found, unlike Bills, that the absorption curve of the substance A was greater than that of ergosterol itself. They believe this may be due to the fact that they measured their solutions sooner and also to

a lack of stirring of the solutions by Bills and others. Substance A was formed by irradiating a 0.1 per cent solution of ergosterol in absolute alcohol. 30 seconds to 10 minutes, removing the unchanged ergosterol by digitonin, evaporating and extracting with ether. The reasons for their belief that substance A is a single substance are as follows:

1. On repetition they always obtained similar spectra, whether in alcohol or in hexane, when the radiation was continued one to 10 minutes. When it was radiated to form substance B, there was a change in absorption.

2. Ergosterol and substance B were most likely to be contaminants of substance A. They believed that the absence of substance B was shown by the small absorption at 240 mμ. The absence of ergosterol was more difficult to prove. From the solubility in alcohol, they judged there was little if any ergosterol present whereas when some of the solutions were subjected to further irradiation, an increase in absorption at wave-lengths 274 and 311 mμ was shown, thus indicating there was, perhaps, some ergosterol present. Having given this evidence to show that A is a single substance they go on to present the following evidence for believing that substance A is vitamin D.

1. In a long series of quantitative comparisons of absorption spectra and antirachitic activity in solutions of substance A, they found a roughly linear relation between antirachitic activity and intensity of absorption at wave-lengths from 310 to 270 mμ. With high activity, there was high absorption and with decreased activity, lower absorption.

2. A 0.01 per cent solution of substance A in a silica cell with nitrogen stirring was exposed to ultra-violet light in which the wave lengths shorter than 265 mμ were excluded by a "light filter" of alcoholic cobalt chloride. A rapid destruction of substance A occurred by conversion of it to substance B, B having little absorption for wave-lengths longer than 250 mμ was partly protected by the filter and produced in considerable concentration. It showed high absorption at 240 mμ and no antirachitic activity. This proves that substance B is not vitamin D.

3. A solution of ergosterol was irradiated for various lengths of time and divided into two portions. In one portion the activity and the absorption were measured without previous removal of ergosterol. In the other, the per cent of ergosterol was estimated gravimetrically by precipitation with digitonin. From this they calculated the absorption of the products of radiation. The antirachitic activity was found to be proportional to the amount of substance A present. Under their conditions

there is a rapid rise in activity in the first 10 minutes, slow rise after 20 minutes and a maximum at 40 minutes, after which there is a fall.

4. Attempts were made to destroy substance A by oxidation and to observe whether the rates of disappearance of absorption at 280 mμ and of antirachitic activity were equal. The effects of bubbling oxygen through a 0.2 per cent alcoholic solution at 76° C. for one hour were too small for certainty. But when the solution was evaporated to dryness, the exposure of the residue to gaseous oxygen at 100° C. produced a more rapid effect. A pale brown solid residue was formed of reduced solubility in alcohol, with reduced antirachitic activity and reduced absorption at 280 mμ. They think this shows that substance A and vitamin D offer marked resistance in alcoholic solution to oxygen at 76° C., but when dried and subjected to oxygen at 100° C. they are (or it is) oxidized with speed.

5. The fifth piece of evidence which they give is that they have never obtained a solution showing strong absorption at 280 mμ, that did not show strong antirachitic activity or a solution of high antirachitic activity without absorption at 280 mμ. Vitamin D may be a substance in substance A.

6. They give a much less direct argument as their sixth. They say that in preparations of A, the antirachitic activity is destroyed rapidly by radiations between 330 and 260 mμ. Therefore, the vitamin D absorption band lies between these. Attempts to produce it from other sterols failed. If two hydrogen atoms are added to one double bond, the substance is not activated. This makes it probable that vitamin D has the three double bonds of ergosterol.

Hence on the assumption that the preparations of substance A are a mixture of one absorbing substance with other non-absorbing products, it is probable that the absorbing substance is vitamin D. The other hypothesis is that vitamin D of enormous biochemical activity is found in small percentage in substance A. The best preparations of substance A are 2 to 4 times as strong as preparations of which the antirachitic action can be detected in doses of 1/100000 mg. per day. The activity of the hypothetical substance is very high indeed.

7. For the last argument they state that when ergosterol is irradiated the absorption increases regularly. In the earliest stages the increase is proportional to the time of radiation. This shows a direct transition from ergosterol to A. The transition from A to B is the same. It is improbable that vitamin D is an intermediate stage. They point out that the pos-

sibility of the formation of vitamin D directly from ergosterol by an independent reaction is not excluded.

They conclude from all this, that there is good reason to believe that the vitamin D absorption spectrum closely resembles that of substance A. It is possible that A is merely a by-product formed at the same time as vitamin D or that D and A are two closely similar substances related by some reversible process such as condensation or polymerization.

The preparations of substance A were colorless, glassy masses which failed to crystallize. There is the question whether the preparations contained a small quantity of a substance of high specific absorption or a large quantity of a substance of lower absorption. The question is of interest since estimates of the absolute activity of vitamin D have depended on assumptions as to quantum efficiency which they feel are of doubtful validity. They believe that the substance A which was formed by irradiating a 0.1 per cent solution of ergosterol for 30 seconds to 10 minutes, to form as little B and C as possible, and precipitating out the unchanged ergosterol with digitonin, is a single substance and probably vitamin D. From their results they calculated that the smallest amount of pure vitamin they should detect is 1.9×10^{-8} gram. This value is very close to the amount reported by Fosbinder, Daniels, Steenbock and Kon (23, 24) in this country but the authors feel that the results are so dependent on conditions that the fact that the calculations agree so closely is of little significance.

Fosbinder, Daniels and Steenbock (23) and Kon, Daniels and Steenbock (24) made a quantitative study of the photochemical activation of sterols and attempted to determine the amount of energy sufficient to produce, when fed, a demonstrable deposition of calcium in a rachitic animal. First working with cholesterol at the 265 mμ wave-length, they found that an energy input of 234 ergs was necessary to give positive antirachitic potency with an exposure of 22.5 seconds. Making use of the quantum theory, they calculated that 3.2×10^{13} quanta were involved. According to Einstein's law of photochemistry, 3.2×10^{13} molecules of vitamin D were synthesized by 3.2×10^{13} quanta during the exposure of 22.5 seconds. The number of gram molecules were then obtained by dividing the number of molecules by the Avogadro number, which gives 5×10^{-11} gram molecule. Then assuming that the molecular weight of the antirachitic material was essentially the same as that of cholesterol (385), the number of grams of vitamin becomes $5\times10^{-11}\times385$ which equals 2×10^{-8} gram. This indicates that 20 billionths of a gram or 20 millionths

of a milligram of vitamin D is sufficient to produce calcium deposition in a rachitic rat.

They feel that the assumption of the Einstein law is justified as a first approximation. The assumption is also made, of course, that all the light absorbed by purified cholesterol is converted into vitamin D. If some of the light were absorbed by material which was not converted into vitamin D, the quantity found by these calculations would be still further reduced.

The work was repeated using purified ergosterol. This time the ergosterol was irradiated at the 256, 265, 280 and 293 mμ lines and the threshold value of activation for all wave lengths was found to be 700–1000 ergs (10×10^{13} to 14×10^{13} quanta). The lower value corresponds to 6×10^{-8} gram of vitamin D. The quantum efficiency of the antirachitic activation of ergosterol is smaller than that given in the earlier paper but the authors feel that these last results are more accurate.

The formation of vitamin D was thus shown to be independent of wavelength over practically the whole range of selective absorption and to be a function of the incident energy. They exposed, too, chemically purified cholesterols, purified by the severe methods which Bills believes do not wholly destroy their activatability and found that the highest amount of energy used, 200,000 ergs, was insufficient to activate them. This caused them to conclude that ergosterol is definitely the "provitamin."

It may be of interest to sum up the properties of ergosterol which are known and the changes which have been shown to take place in them upon irradiation. The melting point of ergosterol has been reported to be anywhere from 135°–183° C. (26) depending upon the purity of the sample and the degree of hydration. Upon irradiation with ultra-violet light, a glassy hard solid of indefinite melting point is obtained which begins to melt at 30° C. (30).

The $[\alpha]_D^{20°}$ in chloroform of ergosterol is $-132°$ according to Tanret (13), Bills (26) and others. The findings for the specific rotation seem to be in better agreement than for the melting point. When ergosterol is irradiated, its specific rotation changes gradually towards the right and after 45 hours, according to Windaus and Linsert (27) is definitely positive. However, when the $[\alpha]_D$ becomes positive, the irradiated substance is no longer active antirachitically.

Ergosterol is slightly soluble in 95 per cent alcohol, to about 0.2 per cent, while the irradiated substance is soluble in its own weight of alcohol (30). Both are insoluble in water (30).

Windaus and Linsert (27) have shown that the molecular weight of the irradiated substance is the same as that of the unirradiated substance and that the number of double bonds is unchanged on irradiation.

Since the ergosterol itself is precipitated by digitonin and the irradiated product is not, the conclusion is that there has been some change in the OH group, probably an isomerization, since the molecular weight is the same (27).

There seems to be some disagreement as to the effect of the solvent on the potency of the irradiated ergosterol. Windaus (28), using cyclohexane, ether, normal benzene and benzol, reported that the influence of the solvent on the activity was small while Bills (29) reported in August 1929 that the solvent had a very great influence on the potency of the product of irradiation. Bills used alcohol, ether and cyclohexane. He also reported that a freely transparent solvent is not always required, for extremely high activation can be obtained in arachis oil, which is a solvent employed in commercial practice. No complete report on this work has been made by Bills.

Some investigations have been made on the irradiation of cholesterol and ergosterol at very low temperatures. Bills and Brickwedde (31) found that if cholesterol were irradiated at the temperature of liquid oxygen ($-183°$), it was about one-tenth as active as when irradiated at room temperature. Webster and Bourdillon (30) obtained similar results with ergosterol. Both concluded that the temperature coefficient of the reaction causing the formation of vitamin D is small. They argue that the reaction must be entirely photochemical and that the activation is due to an isomerization.

The irradiated substance is more stable to air and light than the unirradiated ergosterol. Irradiated ergosterol has been reported as being very active after standing one and a half years in an inactive oil (32). If oxygen is present on irradiation the absorption band at 248 $m\mu$ develops and fades more quickly than when oxygen is absent indicating that oxidation is concerned in the destruction of the substance showing the 248 $m\mu$ absorption band and not in the formation of vitamin D (19).

It seems almost unnecessary to say in conclusion that an enormous amount of work is left to be done on this subject. Successful solution of the many problems involved can come only as a result of the cooperation of the organic chemist, the physical chemist and the biological chemist.

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